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# DIFFERENTIAL INHIBITION AND MODIFICATION OF ECHINODERM DEVELOPMENT BY METHYLENE BLUE

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IT HAS been found by various investigators that methylene blue in certain concentrations increases oxygen uptake in sea-urchin eggs and in other cells.[1] However, the use of methylene blue as an oxidation-reduction indicator on echinoderm embryonic material and on various Protozoa, coelenterates, planarians, annelids, teleosts, and amphibians (Child, various papers) has shown that, with sufficient staining by the oxidized dye, it becomes toxic and even lethal. In view of these data, the question whether methylene blue would accelerate or inhibit echinoderm development seemed of interest.

In 1948 and 1949 echinoderm material was subjected from early cleavage stages to a considerable range of methylene blue concentrations under different conditions as regarded temperature and light. As the experiments of 1948 were being concluded, a paper by Shapiro (1948) appeared, showing that methylene blue and certain other reversible dyes retarded the first cleavage in eggs of the sea urchin, *Arbacia punctulata*. On the other hand, M. M. Brooks (1943) has stated that echinoderm development is accelerated by methylene blue. So far as retardation is concerned, the data of the present paper agree with Shapiro's results, but the paper is primarily concerned with the differential modifications of development by methylene blue under certain experimental conditions, and only incidentally with the retardation.[2]

## MATERIAL AND METHOD

In 1948, at the height of the breeding season, eggs of the sand dollar, *Dendraster excentricus*, were fertilized at the Hopkins Marine Station, Pacific Grove, were brought to Palo Alto with very slight change of temperature, and, except for controls in water, were transferred to different concentrations of methylene blue in 4–8-cell stages at a temperature of 15°–16° C. in darkness, except during examination. After 24 hours, a part of this material was brought to the temperature of a laboratory room, 22°–24° C., in strong diffuse daylight and on a white background. Also, after ex-

[1] Barron, 1929; Barron and Hoffman, 1930; M. M. Brooks, 1941, 1943; Clowes and Krahl, 1936, 1940.

[2] The kindness of the staff of the Hopkins Marine Station in collecting and making the material available and of Dr. Olin Rulon in sharing his *Dendraster* material for the methylene blue work is gratefully acknowledged.

posure to the dye, some of the material was returned to water containing only very slight traces of the dye.

During January and February, 1949, the breeding season of *Strongylocentrotus purpuratus*, eggs of this echinoderm were also subjected to methylene blue. In this case the animals were brought to Palo Alto, where the eggs were fertilized and were transferred to different dye concentrations at 2–4-cell stages or in some lots slightly later in cleavage. Part of this material was kept in a constant-temperature room at 12° C. in darkness, another part was brought at once to a laboratory room with temperature varying from 16° to 22° C., in strong winter diffuse daylight on white background. None of this material was returned to water. A month later, satisfactory material being still available, a new lot of eggs was subjected to the dye in darkness at 15° C. and at the variable temperature of 16°–22° C. in daylight on white background. The containers were finger bowls of the sort widely used for such purposes, with approximately 250 ml. of dye solution or water. All were washed thoroughly in both fresh and salt water before being used and, when in use, were either stacked or covered by glass plates. The numbers of eggs in each bowl were believed to be sufficiently small that any extreme effects of crowding could not appear. However, in most of the dye concentrations used, the animals remained longer at the bottom of the container than did the controls and in some cases became more or less aggregated. These aggregations were distributed by stirring when observed, but they may have played some part in determining the course of development in certain cases to be mentioned below.

It is to be noted that, in the exposures to the dye at the higher temperatures in daylight, two changes in external conditions from the low-temperature exposures in darkness are concerned: first, the increase in temperature, which doubtless increases the metabolism of the material to some extent and probably makes it more susceptible to the dye, and, second, the exposure to daylight, with its possible photodynamic effects. Moreover, the sand-dollar material was exposed to the higher temperature and daylight only after 24 hours in darkness at the lower temperature, while the *Strongylocentrotus* material was brought into daylight and the higher temperature in early cleavage. Also, both lower and higher temperatures were different in the two cases; the sand-dollar material was exposed to strong summer daylight, the sea-urchin material to daylight of midwinter. The two series of experiments do not permit direct comparison, but the results as regards developmental modification are definite. In the light of earlier work with methylene blue and other dyes on various organisms, it was regarded as probable that if a photodynamic effect on development occurred, it would probably be greater at the higher, than at the lower, temperature. The question of photodynamic effect will be considered elsewhere in the paper, but the data of this paper serve chiefly to call attention to its possible occurrence and effect on development. More complete control of both temperature and light intensity is necessary for investigation of this possible factor.

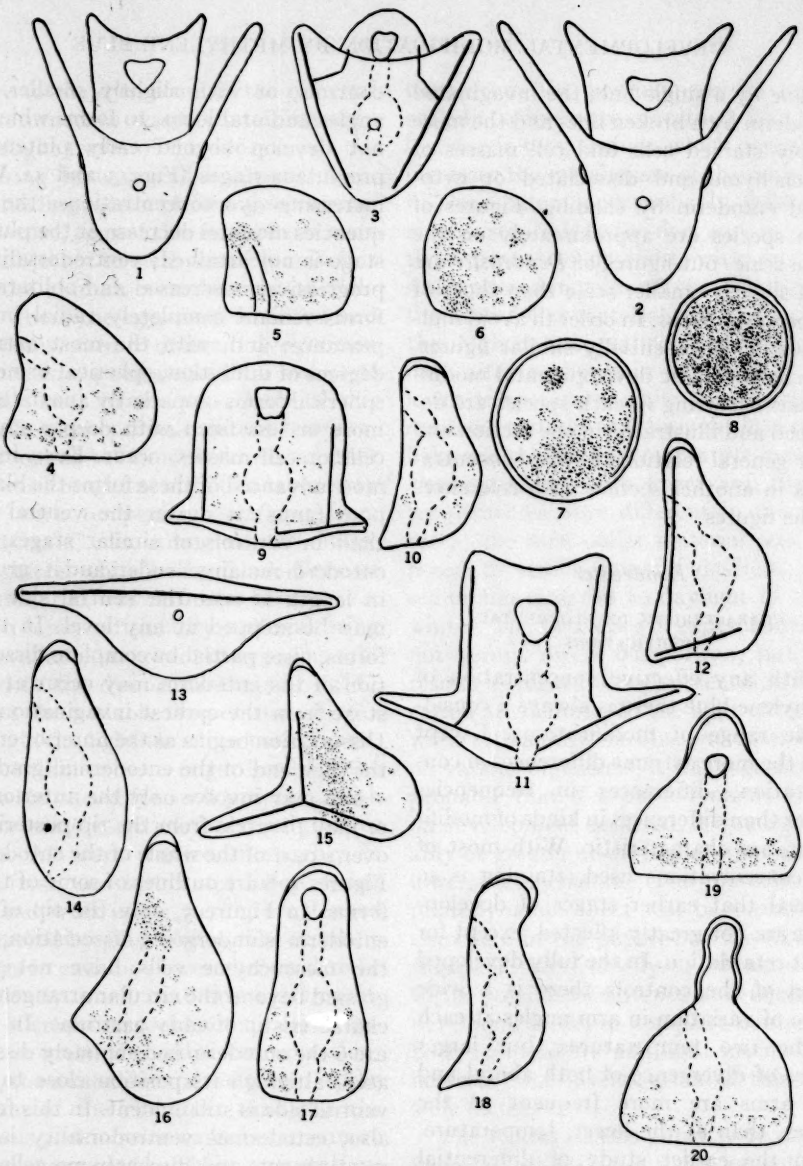
The figures of this paper were drawn from camera lucida outlines, except in a few cases in which individuals of the same sizes as those outlined with the camera were outlined freehand. They indicate the differences in forms and proportions in the various modifications as accurately as possible, but otherwise are diagrammatic. With a few exceptions, the thin ectoderm is indicated merely in

outline by a single line; the invaginated entoderm by a broken line; and the more deeply stained cells and cell masses of mesenchyme and dissociated or cytolyzed entoderm by shading. Figures of each species are approximately on the same scale but figures of *Dendraster* are on a slightly smaller scale than those of *Strongylocentrotus*. In order to avoid multiplication of essentially similar figures, the characteristic developmental modifications occurring in each species are described and illustrated in one section and their general relations to dye concentrations in another section with references to the figures.

## *Dendraster*

### CHARACTERISTIC DEVELOPMENTAL MODIFICATIONS

With any effective concentration of methylene blue there is always a considerable range of modifications. Except with the more extreme differences in concentration, differences in frequencies rather than differences in kinds of modification are characteristic. With most of the concentrations used, staining is so gradual that earlier stages of development are not greatly affected, except for slight retardation. In the fully developed plutei of the controls there is a wide range of variation in arm angles at each of the two temperatures; but larger angles of divergence of both aboral and oral arms are more frequent at the higher, than at the lower, temperature, as in the earlier study of differential modifications by temperature (Child, 1950). Figure 1, a low-temperature form, and Figure 2, a high-temperature form, indicate differences in angles of divergence of arms within the range of higher frequencies in each of the two controls. With slight differential inhibition by the dye, forms range from plutei like the controls, or with slightly smaller arm angles and oral lobes, to forms which do not develop beyond early pluteus or prepluteus stages (Figs. 3 and 4). With increasing dye concentrations, the frequencies of plutei decrease or the pluteus stage is not attained; ventrodorsality is progressively decreased and obliterated; forms remain completely radial in appearance; and, with the most extreme degrees of inhibition, spherical or nearly spherical forms apparently anaxiate and more or less filled with deeply stained cells or cell masses, occur. Even in the more advanced of these forms the blastopore (anus) is nearer the ventral side than in controls of similar stages; the entoderm remains slender; and its growth in length is near the ventral side and may be stopped at any level. In these forms, also, partial or complete dissociation of the entoderm may occur at any stage from the earliest invagination on. Dissociation begins at the anterior end—the high end of the entodermal gradient—and may involve only the anterior tip or may progress from the tip posteriorly over a part of the whole of the entoderm. Figures 5–8 are outlines of some of these forms. In Figure 5, only the tip of the entoderm is undergoing dissociation, and the mesenchyme cells have not progressed beyond the circular arrangement characteristic of early gastrulae. In Figure 6 the entoderm is completely dissociated, although its position close to the ventral side is still evident. In this form, also, ectodermal ventrodorsality is almost absent, and mesenchyme cells are scattered, indicating that the factors which orient them preceding skeleton formation are no longer effective. In Figure 7 gastrulation apparently began, but dissociation of the entoderm began at about the same time, and development did not progress beyond this point. This form showed no evidence of ventrodor-




FIGS. 1–20.—*Dendraster*. Controls and characteristic differential modifications of the methylene blue series. Figs. 1 and 2, controls in water as seen aborally: Fig. 1, characteristic of the lower temperature, Fig. 2 of the higher. Figs. 3–8, various degrees of differential inhibition in methylene blue: Fig. 3, slight differential inhibition, as seen aborally; Figs. 4–6 more extreme differential inhibitions, viewed laterally, showing progressive decrease of ventrodorsality and occurrence of entodermal dissociation; Fig. 7, no evidence of ventrodorsality, and the only evidence of polarity is slight elongation and mass of dissociated cells at one pole; Fig. 8, apparently anaxiate. Figs. 9–18, secondary modifications resulting from decrease in concentrations of intracellular dye in the dye solutions or after return to water and differential recovery in development or perhaps in some cases a differential acclimation. Figs. 9 and 10, 11, and 12 are ventral and lateral outlines of the same two individuals; Figs. 13 and 14, aboral and lateral outlines of a single individual; Figs. 15, 16, and 18 are lateral, Fig. 17 a ventral, outline of other cases. Figs. 19 and 20, forms seen only occasionally, suggesting entodermization of apical region.

sality in any position. Figure 8 is apparently completely anaxiate. Forms like Figures 7 and 8 may remain more or less motile, but the motility seems to be without direction. In these more extreme inhibitions, mesenchyme and entoderm remain deeply stained.

The forms of Figures 5–8 represent samples from a graded series of differential inhibitions. The apical region of the early embryo, from which the oral lobe develops, and the ventral region, on which the mouth and the aboral arms of the pluteus develop, representing the regions of higher susceptibility and of more rapid oxidation and reduction of indicator dyes, are progressively more inhibited than are other regions, with decrease and obliteration, first, of ventrodorsality and, finally, of polarity, as an effective factor in morphogenesis. In the entoderm, progressive inhibition and dissociation show a similar relation to the entodermal gradient pattern. This series of differential inhibitions is not essentially different, as regards their relation to the gradient pattern, from those produced earlier by other external agents.[3]

With continuing exposure to the lower dye concentrations used, e.g., 1/300,000, 1/200,000, the developmental stages become slightly stained at first, but, as development progresses, staining decreases gradually, and they may become almost or entirely colorless. They either reduce the intracellular dye or destroy it in some way. This decoloration is apparently not due to dye reduction, since no return of color in any case has been observed with increase of oxygen content of the solutions. A further point of interest in this connection is that, with a relatively large number of animals in low dye concentration, a progressive decrease of color in the solution has been observed, although depth of staining of the animals did not increase and might even decrease. The ability to decolorize the intracellular dye seems to increase during development. This may be regarded as a process of acclimation. On return to water after 24 hours or more in dye solutions from early stages, more or less decoloration occurs, depending on concentration of the dye and depth of staining. With increasing dye concentrations, the decoloration is largely or entirely ectodermal, entodermal and mesenchyme cells remaining deeply stained, often with more or less dissociation of the entoderm.

With this decoloration there is a decrease of toxicity and of inhibiting effect, both with continuous exposure to the dye and after return to water. Under these conditions secondary developmental modifications occur, opposite in character to the differential inhibitions (Figs. 9–20). In the more advanced of these modifications the apical region develops into a relatively large, and usually more or less elongated and thickened, outgrowth, which represents an early stage of development of the oral lobe but does not undergo co plete differentiation and development of oral arms (Figs. 9–12, 14–18). In these forms the aboral arms are short—mere buds—or may be completely absent; but, when they are present, the angles of divergence are large and may approach or reach 180° (Figs. 9, 11, and 13). The ventral region is relatively large and only slightly concave or almost flat instead of rather deeply concave, as in the usual pluteus form,[4] and in the more extreme cases the body is flattened ventrodorsally (Figs. 9, 10, 11, 12, 13, 14), and the tip of the oral lobe is often recurved toward the dorsal side (Figs. 14 and 18), apparently as a result

[3] Child, 1941, pp. 199–204 and literature cited there, and 1948; Rulon, 1948, 1950*a*, *b*.

[4] Figures 45, 46, 47, and 59, 60, 61 (Child, 1950) show variations in this concavity in the usual pluteus form at different temperatures.

of relative overdevelopment of the ventral region. In Figure 13, drawn as seen from the anal side, the larva is so much flattened that the oral lobe is not visible. Figure 14 is an outline of the same larva in side view. In these secondary modifications the entoderm may be intact (Figs. 9, 10, 11, 12, 14, 17, 18), dissociated anteriorly, or completely (Figs. 15 and 16).

These forms result from the less extreme degrees of differential inhibition in earlier stages, followed by some degree of differential acclimation or of differential recovery from the toxic effects associated with decoloration in the solution or with return to water. In all of them, these modifications consist of relative overgrowth and progress of development of those regions of the gradient pattern which oxidize and reduce indicators more rapidly and are more susceptible to the action of external agents. If not too deeply stained, these regions are able to dispose of methylene blue more rapidly and completely than other regions in low dye concentrations and to recover more rapidly and completely from differential inhibition after return to water. Entoderm and mesenchyme are less able than ectoderm to dispose of the dye, but, in low dye concentrations and following return to water, they may also undergo more or less decoloration. Dissociating or dissociated entoderm may retain in some degree the position of the living entoderm (Figs. 15 and 16), or, with earlier and more complete dissociation, the cells or cell masses may become scattered with mesenchyme cells throughout the blastocoel, and, in some cases of inhibition, dissociation evidently occurs with the beginning of invagination and both entoderm and mesenchyme apparently immigrate.

Secondary modifications with unsegmented or segmented apical outgrowth (Figs. 19 and 20) occurred occasionally in some of the lots exposed to dye. They are like those produced by $NaN_3$, LiCl, and various other conditions in *S. purpuratus* (Child, 1948) but have not been previously observed in the sand dollar. They appear, at least in the more extreme forms, to represent entodermization of the apical region in *Strongylocentrotus*, and the segmentation (Fig. 20) and thickening of the wall suggest that they have the same significance in the sand dollar. Exogastrulae, ranging from forms with well-developed pluteus ectoderm and evaginated entoderm to smaller, completely radial exogastrulae, with entodermization of most of the ectoderm, appeared in some lots in the lower dye concentrations in which there was more or less decoloration in later stages and in some lots returned to water, but not above an estimated 5–10 per cent. No attempt was made in these experiments to determine optimal conditions for their development. From earlier work and since it has become evident that exogastrulae are not a specific effect of LiCl or any other agent, it appears evident that they represent certain degrees of differential inhibition in early development, followed by some degree of secondary modification by acclimation or recovery, at least in the more extreme forms with very large entoderm. Crowding of animals in a container is a very effective way of producing exogastrulation. In methylene blue the earlier stages remain at the bottom of the container longer than do the controls, and in some cases they become aggregated. The possibility is noted that such aggregations, happening to occur at the right stage of development, may have played some part in determining an occasional exogastrula.

### DEVELOPMENT IN RELATION TO DYE CONCENTRATION AND OTHER CONDITIONS

In methylene blue 1/300,000 at 15° C. in darkness, staining was slight in earlier

stages, and most individuals gradually became almost or quite colorless in the dye solution after 2–3 days, except for scattered mesenchyme, which remained stained. An estimated 80–90 per cent became plutei two-thirds to three-fourths the size of the controls, with a higher frequency of large oral lobe and large aboral-arm angles but with aboral arms shorter than in controls. The short aboral arms suggest that the activation of the regions of aboral-arm development was still somewhat inhibited. In the remaining 10–20 per cent there was evidence of secondary modification: ventral regions were relatively or absolutely larger and flattened, aboral arms were short, and the oral region was somewhat elongated. These forms resembled Figures 9–17 above, except that entodermal dissociation was not observed in any case. In short, almost every individual in this dye concentration showed evidence of some degree of secondary developmental modification characteristic of differential acclimation or differential recovery, following very slight differential inhibitions.

With higher concentrations of dye (1/200,000–1/50,000), frequencies of the different forms in different containers were somewhat variable, but the effects of the dye were evident. In 1/200,000 dye at 15° C. in darkness, staining occurred during the first 2 days and was followed by partial to almost complete decoloration. The forms ranged from 25–50 per cent of small plutei with short aboral arms and with a high frequency of somewhat elongated oral lobes, i.e., showing some degree of secondary modification resulting from the considerable decoloration, through the forms of Figures 9–18 with greater degrees of secondary modification and an occasional exogastrula and a few much more inhibited individuals like Figures 5–8. The small numbers of these cases of extreme inhibition in this and higher concentrations and the considerable difference in their condition and that of the very great majority of the lots suggest that other conditions than the dye may have been concerned in their occurrence; perhaps a few somewhat defective eggs—a frequent occurrence in cultures—may have been further inhibited by the dye. In the secondarily modified forms, which did not develop skeleton, mesenchyme cells were more or less scattered singly or in masses, but some degree of ventral aggregation was still visible in many individuals. These cells did not undergo appreciable decoloration. In individuals of the secondarily modified types, skeletal anomalies were frequent in those with skeletal development. In general, there was more decoloration in individuals showing the greater degrees of secondary modification.

After 24 hours in darkness at 15° C., a lot was transferred to a temperature of 22°–24° C. in strong diffuse daylight on white background. These stained at first but later underwent almost complete or complete decoloration, including mesenchyme cells in most individuals, though a few retained some color in the mesenchyme. They showed all degrees of secondary modification (Figs. 9–20), with an occasional exogastrula and again a few extreme inhibitions (Figs. 7 and 8), as in the same concentration at low temperature in darkness. With the change to high temperature and light and with the more complete decoloration apparently associated with it, virtually the entire culture showed secondary modification. The greater degree of decoloration is probably associated with the increase in metabolism at the higher temperature.

In 1/100,000 at the low temperature in darkness, deeper staining and somewhat less decoloration occurred. The resulting forms were almost all small plutei, about half the size of the controls but with a high frequency of relatively long

oral lobe and large angles of divergence of aboral and oral lobe; but only an estimated 5 per cent showed any marked ventral flattening. Cells in the region of the ciliated band and often in the ventral region, including the ventral side of the oral lobe, remained somewhat stained in many individuals. Here also a few spherical, apparently anaxiate, individuals occurred; as noted above, they are so widely different from the rest of the culture that it seems probable that they are not due to methylene blue alone. These forms were almost all differentially inhibited, with only slight secondary modification.

In 1/50,000 at low temperature in darkness, staining became deep, and early development was much more retarded than in the lower concentrations. Even after 48 hours, immigration of mesenchyme was occurring or had not begun. In an estimated 30–40 per cent of the lot the entodermal region was somewhat enlarged and thickened and protruded, as in early stages of exogastrulation, but only a few individuals became exogastrulae. The animals continued to stain without any appreciable decoloration. However, an estimated 80–90 per cent became small plutei, half, or less than half, the size of the controls, with small oral lobes, short aboral arms, and small arm angles. The remainder did not attain the pluteus stage, but extreme inhibitions like Figures 7 and 8 were still only few in number. These lots showed only differential inhibition.

Material in 1/50,000 dye, transferred after 24 hours from the lower temperature and darkness to 22°–24° C. in strong diffuse daylight on white background during the day, also became deeply stained but in later stages showed slight decoloration in some individuals. The resulting forms were preplutei, i.e., forms about to develop aboral arms; inhibited gastrulae, with high frequencies of partly or wholly dissociated entoderm (Figs. 5–7); and a higher frequency of spherical, apparently anaxiate, forms more or less filled with cells (Fig. 8) than at low temperature in darkness in this or lower concentrations. This dye concentration was distinctly more toxic at the higher temperature in daylight than at the low temperature in darkness. The possibility that the increased metabolism at the higher temperature increases the susceptibility to the dye is noted, but the increase in degree of inhibition appears greater than is to be expected from the few degrees of change in temperature. Probably a photodynamic effect is concerned in the increase of inhibition at the higher temperature in daylight.

After 48 hours in 1/50,000 dye at the low temperature in darkness at, or shortly before, immigration of mesenchyme, a lot was transferred to water and continued under the same external conditions. Decoloration became almost or quite complete in this lot, except for cells and cell masses in the blastocoel, which retained some color. An estimated 50–60 per cent became plutei, within the normal range of forms, smaller than controls but larger than in lots remaining in the dye under the same external conditions. The remainder showed all degrees of differential recovery in their morphogenesis (Figs. 9–18), with a high frequency of partial or complete entodermal dissociation and with a few individuals showing more extreme degrees of inhibition without recovery (Figs. 5–8).

After 24 hours at 22°–24° C., following 24 hours at the lower temperature, a part of the material was transferred to water and continued at 22°–24° C. in diffuse daylight on white. In water, different degrees of decoloration occurred. A few of the less inhibited individuals became almost completely colorless, except

for the cells in the blastocoel. Others showed less decoloration, often with less decoloration in the oral lobe than elsewhere, suggesting slight injury of this most susceptible region of the larva by the dye. Development in this lot ranged from an estimated 5–10 per cent of plutei, approaching the controls in form and proportions, through the entire range of differential recoveries (Figs. 9–18) and, as noted in other series described above, a few individuals showing the extreme degrees of differential inhibition (Figs. 7 and 8). As in other cases, the forms approaching the controls and those with the higher degrees of differential recovery have undergone, in general, more decoloration. In this lot at the higher temperature in daylight both before and after return to water, the dye is more toxic than in the same dye concentration at the lower temperature in darkness both before and after return to water. Here only an estimated 5–10 per cent became plutei within the normal range of form, as compared with 50–60 per cent in lots continuously at the lower temperature in darkness. This difference also suggests a slight photodynamic effect in daylight.

### *Strongylocentrotus*

#### CHARACTERISTIC DEVELOPMENTAL MODIFICATIONS

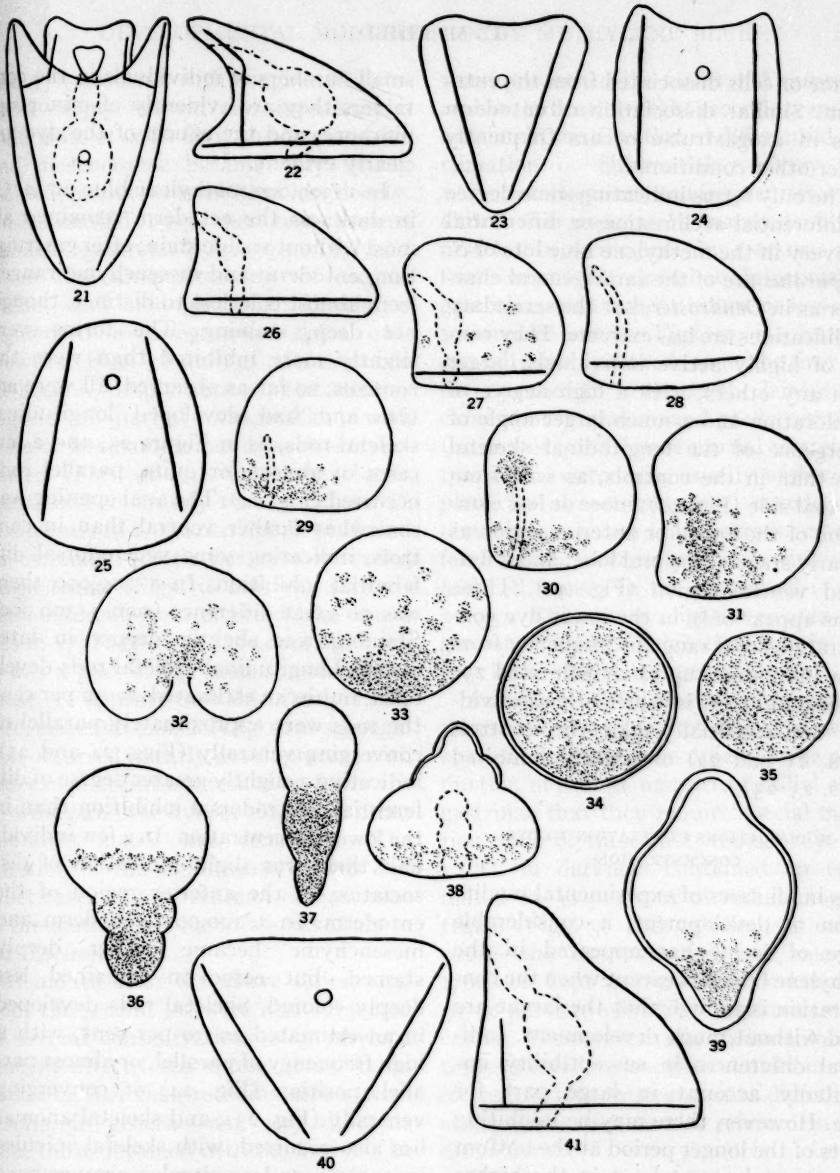
*Strongylocentrotus* is very much more susceptible to injury by external agents than is *Dendraster*. In several cases the animals have been transported from Pacific Grove to Palo Alto with almost no temperature change and no drying, and some animals have been kept overnight in a refrigerator before fertilizations were made. Fertilizations following these procedures are usually virtually 100 per cent; but, when the animals are kept for longer periods without running water, the percentages of fertilization usually decrease. However, although there appears to be no retardation or modification of earlier development with these procedures, the plutei developing in filtered sea water, either fresh or kept in the laboratory after filtering, show a slight degree of differential inhibition, which is independent of temperatures between 12° and 22° C. and occurs in darkness as well as in daylight. The fully developed pluteus stage attained without food is very similar to the pluteus of *Dendraster*, though somewhat smaller. During experiments of several years, the characteristic pluteus form attained in controls with the procedures noted above is outlined in Figures 21 and 22. Development of the aboral arms has stopped at an early stage, and their angle of divergence is rather small, being slightly above 30° in Figure 21. Also, the oral lobe is small and not fully differentiated, but the larvae remain active about as long as *Dendraster* plutei. These pluteus forms are differentially inhibited to a slight degree. Oral lobe and ventral regions—the high regions of the gradient pattern—are most inhibited, and the more dorsal regions are relatively large. The aboral arms, which represent gradients developing from regions of activation appearing in later development are also inhibited. Evidently this slight inhibition of pluteus development, whatever the factors determining it, is differential, with the same relation to the gradient pattern of the animal as that of inhibitions by many other agents.

In marked contrast to the results obtained with *Dendraster*, the developmental modifications by methylene blue are almost entirely differential inhibitions, varying widely in degree. The least-modified forms attain almost the stages of the controls. Longitudinal skeletal rods develop, with small angles of divergence (Fig. 23) or frequently parallel (Fig. 24)

and occasionally converging ventrally (Fig. 25); but arm development does not occur. Seen from the side, these forms are similar to Figure 26. A characteristic feature of these forms is the location of the anal opening (blastopore) nearer the ventral side than in uninhibited forms, i.e., the ventral region is more inhibited in development than are the more dorsal regions, which remain rounded and relatively large (Figs. 24 and 25). Development of the oral lobe from the apical or anterior end of the larva is completely inhibited, and the secondary activation from which the aboral arms develop is almost or entirely absent. With slightly greater degrees of differential inhibition, the anus-blastopore is still nearer the ventral side (Fig. 27) and the entoderm may be very slender and without regional differentiation (Fig. 28), or invagination may stop before completion (Fig. 29). With further increase of the effect of the dye, all degrees of dissociation of entoderm occur, progressing from the anterior end. This may involve only the anterior tip (Fig. 30), may stop at any level, or may progress over the whole entoderm (Fig. 31). In all the forms noted thus far, differential inhibition has decreased but has not obliterated ventrodorsality. The change from the radial to the ventrodorsal form has occurred, but ventral, as well as anterior, development has been increasingly inhibited.

With more extreme differential effects of the dye, ventrodorsality never becomes evident in morphogenesis. The forms remain completely radial, and invagination is usually only partial, with early and almost or quite complete entodermal dissociation (Figs. 32 and 33). In these forms there is still an indication of the polar pattern in the location of the inhibited invagination or of the mass of dissociated or dissociating cells. In the most extreme degrees of differential inhibition there is no evidence of either ventrodorsality or polarity or of differentiation of ectoderm and entoderm. These forms are the "blastulae," more or less completely filled with cells and cell masses, as observed by many investigators under many different conditions. They differ in size according to the developmental stage attained (Figs. 34 and 35). Whether the cells and cell masses dissociate from the regions of prospective mesenchyme and entoderm or more generally from the wall remains uncertain. These forms may remain more or less motile for some days but apparently do not show definitely directed movement. In *Strongylocentrotus* material these forms occur in such numbers and under such conditions that there is no doubt of their production by methylene blue. This point is noted by way of contrast with the *Dendraster* material, in which they appear only in very small numbers and are so different from the rest of the cultures concerned as to suggest that they represent unusually susceptible or perhaps somewhat defective eggs.

In many lots in the dye, completely radial exogastrulae (Figs. 36 and 37) appeared, in frequencies from a few individuals up to 50–60 per cent (estimated) of the lot. Also exogastrulae or entogastrulae with unsegmented or segmented apical outgrowths occurred occasionally in certain lots (Figs. 38 and 39). These are similar to the forms described in an earlier paper (Child, 1948) on *Strongylocentrotus* modifications produced by sodium azide and other inhibiting conditions. In *S. purpuratus* exogastrulation occurs very readily under many different conditions, and it is of interest to find that methylene blue may also be concerned in determining it. In the methylene blue exogastrulae, the evaginated entoderm appears in almost all cases to be a solid mass of cells, either mesen-




FIGS. 21–41.—*Strongylocentrotus*. Controls in water and characteristic differential modifications in methylene blue. Figs. 21 and 22, controls, slightly inhibited in later pluteus development. Figs. 23–25, slight differential inhibitions by dye, viewed aborally, showing change from divergence to parallelism and convergence ventrally of longitudinal skeletal rods. Fig. 26, lateral outline of forms like Figs. 23–25. Figs. 27–31, increasing degrees of differential inhibition seen laterally, showing decrease in ventrodorsality and in entodermal differentiation, and occurrence of entodermal dissociation. Figs. 32–34, no evidence of ventrodorsality; polarity still indicated by position of largest masses of dissociated cells. Fig. 35, apparently anaxiate. Figs. 36 and 37, completely radial exogastrulae, frequent in certain lots. Figs. 38, and 39, ento- and exogastrula, with segmented or unsegmented apical outgrowth, frequent in certain lots. Figs. 40 and 41 are the only modifications indicating differential recovery.

chyme or cells dissociated from the entoderm. Similar dissociation of entoderm cells in exogastrulae occurs frequently under other conditions.

The only forms indicating some degree of differential acclimation or differential recovery in the methylene blue lots of *S. purpuratus* are of the same general character as in *Dendraster*, but the secondary modifications are less extreme. They consist of highly active individuals, larger than any others, with a high degree of decoloration and a much larger angle of divergence of the longitudinal skeletal rods than in the controls, as seen from the anal side (Fig. 40); more or less elongation of the apical or anterior region as an early stage of the oral lobe; and a flattened ventral region (Fig. 41). These forms appear only in the lower dye concentrations and range in frequency from a few individuals up to an estimated 25–30 per cent in lots in which other individuals are essentially like the controls (Figs. 21 and 22) or slightly inhibited (Figs. 23–25).

### MODIFICATIONS IN RELATION TO DYE CONCENTRATION

As in all cases of experimental modification of development, a considerable range of forms has appeared in the methylene blue lots except when the concentration is so high that the larvae are killed without much development. Individual differences in susceptibility undoubtedly account in large part for these. However, there may be inhibiting effects of the longer period at the bottom of the container, at least in the higher dye concentrations and the resulting greater probability of aggregations, both these conditions interfering to some extent with oxygen supply and perhaps with removal of $CO_2$. These conditions may play some part in determining frequencies of particular forms, but with the small numbers of individuals in the containers they are evidently of minor significance, and the effects of the dye are clearly evident.

In 1/300,000 methylene blue at 12° C. in darkness the ectoderm remained almost without visible stain; after gastrulation, entoderm and mesenchyme ranged from almost colorless to distinct, though not deep, staining. The forms were slightly more inhibited than were the controls; so far as observed, all were active and had developed longitudinal skeletal rods, as in Figure 23, and a few cases of almost, or quite, parallel rods occurred (Fig. 24). The anal opening was somewhat farther ventral than in controls, indicating some ventrodorsal differential inhibition. In 1/200,000 there was no great difference from 1/300,000. Staining was slightly deeper in later stages. Longitudinal skeletal rods developed, but in an estimated 25–30 per cent the rods were approximately parallel or converging ventrally (Figs. 24 and 25), indicating a slightly greater degree of differential ventrodorsal inhibition than in the lower concentration. In a few individuals there was slight indication of dissociation in the anterior region of the entoderm. In 1/100,000, entoderm and mesenchyme became rather deeply stained, but ectoderm remained less deeply colored. Skeletal rods developed in an estimated 20–30 per cent, with a high frequency of parallel, or almost parallel, position (Fig. 24) or converging ventrally (Fig. 25); and skeletal anomalies also occurred, with skeletal spicules in a more or less circular arrangement around the entoderm. In these cases the mesenchyme had not lost its earlier circular arrangement. An estimated 60 per cent did not develop beyond the stages of Figures 27–31, and, in these, anterior dissociation of entoderm was frequent. A few exogastrulae, radial in form (Figs. 36

and 37), occurred with evaginated entoderm deeply stained and apparently a solid mass of cells. In 1/50,000 entoderm and mesenchyme became very deeply stained, ectoderm somewhat less deeply. An estimated 25–30 per cent became radial exogastrulae (Figs. 36 and 37), and about the same percentage of exogastrulae and entogastrulae developed segmented or unsegmented apical outgrowths (Figs. 38 and 39). In 1/20,000 all became deeply stained, but various degrees of partial invagination, with more or less entodermal dissociation, occurred (Figs. 30–33). One other lot is mentioned separately because, at 12° C. in darkness in 1/250,000 dye, it produced an estimated 50–60 per cent of radial exogastrulae (Figs. 36 and 37), a result very different from either of the above lots in 1/300,000 or 1/200,000, although it was a part of the same lot of eggs as those. It appears probable that, in this case, either an unusual degree of aggregation occurred in early development, with the exogastrulation resulting from the crowding and the dye, or that some other unknown factor was concerned. The question of the significance of this and other cases of occurrence of exogastrulae in the methylene blue lots is taken up in the discussion.

A month after the above series, eggs from the second lot were also subjected to several concentrations of methylene blue in filtered sea water at 12° C. in darkness, like the preceding series, with results, in general, not very different from those obtained with the first lot. In 1/300,000 there was only slight differential inhibition. All developed longitudinal skeletal rods, as in material of the first lot, but in an estimated 50 per cent the rods were almost parallel or were parallel or converging ventrally (Figs. 23–25), as compared with 25–30 per cent in the first lot. A few individuals in this concentration approached the forms of Figures 40 and 41, indicating slight secondary modification in the direction of differential acclimation or differential recovery. In 1/120,000 the forms and frequencies were almost the same as in material of the first lot in 1/100,000. In 1/60,000 the forms were ventrodorsal or almost radial gastrulae, with intact or more or less dissociated entoderm (Figs. 27–31), but no exogastrulae appeared, as in the first lot in 1/50,000. In 1/20,000 the larvae became almost opaque, and 70–80 per cent (estimated) underwent partial invagination of the entoderm, usually with complete, or almost complete, entodermal dissociation; showed no ventrodorsal differentiation; or were spherical, with cells and cell masses in the blastocoel (Figs. 32–35). This was a slightly greater degree of differential inhibition than in the first lot in this concentration.

Animals from the second lot of eggs in three concentrations of the dye were again so different from others in the production of a large number of radial exogastrulae that they require special mention. One of these in 1/160,000 dye at 12° C. in darkness contained an estimated 25–30 per cent of radial exogastrulae (Figs. 36 and 37). In two other cases—one in 1/100,000 and one in 1/60,000 kept at 15° C. in darkness—50–60 per cent of radial exogastrulae occurred. These cases raise the same questions concerning exogastrulation in the methylene blue experiments as did others noted above and are also referred to the discussion.

The development of *Strongylocentrotus* in methylene blue at the higher variable temperature (air temperature ranging from about 16° C. at night to 22° C. during the day and exposed to winter diffuse daylight on white background) shows certain points of interest as compared with the development at 12° C. in dark-

ness. In 1/300,000, the ectoderm remained almost or quite colorless or lost color after very slight staining in early stages; the entoderm and mesenchyme showed only slightly more color. All individuals observed developed longitudinal skeletal rods (Fig. 23), and very few approached parallelism. An estimated 25–30 per cent were like Figures 40 and 41, indicating secondary modification. This is the only case in which these modifications, indicating some degree of differential acclimation or differential recovery, occurred in considerable numbers in *Strongylocentrotus*. In general, they seemed to show less evidence of staining than did others. In 1/250,000 there was no marked difference from 1/300,000, except in the absence of the large, secondarily modified forms. In 1/200,000 a difference from the low-temperature–darkness forms began to appear. Staining appeared to be deeper than at the low temperature, and only 2–3 per cent (estimated) developed longitudinal skeletal rods, as compared with almost all in the low-temperature–darkness lot. The remainder were forms like Figures 28–35, with a very few radial exogastrulae. Even in a few individuals with longitudinal skeletal rods, the entoderm was completely dissociated. In 1/150,000, staining was still deeper, without distinct evidence of decrease in later stages. A few individuals attained the stage of Figure 28, others only those of Figures 29–35. No lots were exposed to this concentration at the lower temperature. In 1/100,000, staining was deep throughout, and 20–25 per cent became radial exogastrulae (Figs. 36–37), usually with more or less dissociating entoderm externally; others were forms like Figures 32–35, without ventrodorsality and in the more extreme forms without evidence of polarity. In this lot no individual with any visible evidence of ventrodorsality was observed. In the low-temperature–darkness lot in this concentration, 20–30 per cent developed skeletal rods, and others were like Figures 27–31, with a few exogastrulae, i.e., ventrodorsality was present in all except the exogastrulae. In daylight at the higher-temperature development in 1/50,000 dye, 20–25 per cent of radial exogastrulae occurred, with entoderm dissociating externally, also a few individuals like Figures 32 and 33 and all others apparently anaxiate, like Figures 34 and 35. In the low-temperature–darkness lot there were also 25–30 per cent exogastrulae and about the same percentage of apical outgrowths; the others were ventrodorsal forms like Figures 27–31. In 1/20,000 in daylight, all were killed at the beginning of gastrulation or earlier; in the low-temperature–darkness lot, partial invagination occurred, and the larvae remained alive for a week or more.

In short, the two series in darkness and in daylight at the two temperatures differ very little in the low concentrations of dye, but, with increasing concentration, the degrees of differential developmental inhibition and lethal effect increase very greatly. Also staining becomes deeper in the daylight series. The question whether this increase in toxicity of the dye with increase of concentration in daylight indicates a photodynamic effect is taken up in the general discussion.

The following few data are of some interest in relation to the occurrence of exogastrulation. In certain lots of eggs fertilized after the urchins had been kept overnight in a refrigerator, there was only about 50 per cent fertilization. In two lots from these—one in 1/100,000 and the other in 1/60,000 dye and kept in daylight on white at the higher variable temperature—an estimated 50–60 per cent of exogastrulae occurred, all small and completely radial. In this case

the physiological condition of the eggs or sperm may have been concerned in the result. In three of the eleven controls of *Strongylocentrotus* in water, there was some differential inhibition beyond the slightly inhibited plutei of Figures 21 and 22 to forms like Figures 23–27, but in these only a single case of very slight exogastrulation was found. This is perhaps further evidence that the dye is an important factor in determining exogastrulation in the methylene blue lots.

## DISCUSSION

Since methylene blue has been shown by various investigators to increase respiration in sea-urchin eggs, as was noted in the introduction, and since M. M. Brooks (1943) has stated that it accelerated development, its action on the course of echinoderm development, both in differential inhibition and also in the secondary modifications of acclimation and recovery with the procedures described above, acquires a particular interest. Shapiro (1948), discussing the retardation of the first cleavage by this and other dyes, suggests that the increase in respiration involves a dissipation of energy from its usual relation to development.

According to the preceding data, methylene blue retards echinoderm development differentially, resulting in differential modifications of development and, with sufficient intracellular concentration, in differential lethal effect. Moreover, these effects of the dye show the same general relation to the gradient pattern of the embryo and larva as do those produced by many other inhibiting agents. The apical or anterior region, from which the oral lobe develops, and the so-called "ventral" region are the regions which oxidize and reduce oxidation-reduction indicators most rapidly, are most susceptible to toxic effects of many external agents, and are also the most highly modified in development. In differential inhibition these regions are most inhibited with decreasing degrees of inhibition at lower gradient levels, and the regional differences of polarity and ventrodorsality are progressively decreased and obliterated. In the secondary modifications of differential acclimation and differential recovery, the regional axial differences are increased.

Organisms, very generally, are more or less capable of gradually ridding themselves of intracellular methylene blue and various other "vital" dyes in the lower concentrations, but with increase of intracellular concentration beyond a certain point, which differs with different forms, this ability decreases and disappears, and the dyes become increasingly toxic and finally lethal. *Strongylocentrotus* is much more susceptible to differential inhibition by the dye than is *Dendraster*, as it is to other external toxic agents generally. The developmental stages of *Strongylocentrotus* do rid themselves of the dye in the lower concentrations and show slight degrees of differential recovery (Figs. 40 and 41) but are apparently less capable than *Dendraster* of gradual decoloration in the higher concentrations. It has already been noted that the gradual decoloration in the same solution in which primary staining occurred is apparently not a reduction of the dye but a decoloration and destruction in some other way. In *Dendraster* the decoloration occurs more rapidly and to a greater degree in daylight at the higher temperature. This is probably also the case in *Strongylocentrotus*, since it is only under these conditions that differential recovery occurs to any considerable degree (Figs. 40 and 41). It is possible that the increase of metabolism at the higher temperature is a factor in the greater decoloration at this temperature, but the

possibility of a photodynamic effect must also be noted as a possible stimulant or excitant. This possibility will be considered more fully below. Whether the decoloration itself increases respiration is another question which remains unanswered. It seems possible that it may do so, since recovery from an inhibiting factor may increase respiration. Moreover, it seems possible that the process of destruction of the dye may have this effect. Mesenchyme and entoderm, after immigration and invagination or evagination, are much less capable of decolorizing the dye than is ectoderm, either because of earlier injury by more rapid staining or in consequence of differentiation. In both forms with sufficiently low concentrations of dye, ectoderm may remain virtually colorless or may become colorless after primary staining and in *Dendraster* may show considerable differential recovery as regards form and regional development, while entoderm and mesenchyme remain deeply stained and entoderm may undergo partial or complete dissociation and become, like mesenchyme cells and cell masses, scattered in the blastocoel and deeply stained. Evidently, entoderm and mesenchyme have become very different from ectoderm in their ability to rid themselves of the dye. This great difference has not been observed in stages before immigration and gastrulation. However, as regards the entoderm, it appears in the evaginated entoderm of exogastrulae. In relation to this point it is of some interest that entodermal dissociation occurs in *Dendraster* and in the asteroid *Patiria* in LiCl and various other inhibiting conditions and that it may occur in both invaginated and evaginated entoderm (Child, 1940). Even as regards this dissociation, methylene blue does not differ in effect from various other inhibiting agents. This difference between ectoderm and entoderm suggests progress of differentiation, but ectodermal modifications of development and the gradient of entodermal dissociation indicate that gradient pattern is still an important factor in both.

The frequent occurrence of exogastrulae in methylene blue—in *Dendraster* only in small numbers, in *Strongylocentrotus* in numbers ranging from a few individuals up to an estimated 50–60 per cent of the number in a container—is of interest, although it is an incidental feature of the work with the dye. In most cases the exogastrulae have developed in the higher dye concentrations, but, in one container in 1/250,000 at the low temperature in darkness, half or more of the larvae were radial exogastrulae. In other lots of the same series in concentrations both below and above this, there was only slight inhibition, with an occasional exogastrula or none. Exogastrulation is now known to occur under a great variety of external inhibiting conditions. It apparently results from certain degrees of differential inhibition occurring rather rapidly in the earlier stages of development and probably reversing the gradients between outer and blastocoel surfaces of entoderm cells, and, if entodermization of ectoderm also occurs, the evaginated entoderm may become very large. After return from the inhibiting agent to water, more or less differential recovery may occur.

Crowding of animals in a container is an effective way of producing exogastrulation, particularly in *Strongylocentrotus*, but is much less effective, unless extreme, in *Dendraster*. As noted earlier, it was believed that the number of animals in a container was low enough to avoid effects of crowding. However, unless the containers are continuously shaken or stirred, aggregations do sometimes occur before the larvae become free swimmers;

also in the higher concentrations of the dye used they remain at or near the bottom longer, and aggregations are more likely to occur than in water. The filtered water used was the same in all cases, and the containers were thoroughly washed in fresh and salt water before being used. The high frequency of exogastrulae, noted in an earlier section, in 1/100,000 and 1/60,000 dye from eggs and sperm from animals kept overnight in a refrigerator without water and with only 50 per cent fertilization suggests that the physiological condition of eggs or sperm may be a factor in determining exogastrulation. In various other lots, eggs and sperm were not always taken from single individuals, and differences in susceptibility to the dye or other physiological differences may have been a factor in determining exogastrulation. However, the absence of exogastrulae, except for a single individual, in several inhibited control containers of *Strongylocentrotus* seems to indicate that the dye was an important factor in determining the development of high frequencies of exogastrulae, though the physiological condition of the embryos and incidental aggregations may play a part in *Strongylocentrotus*. In the much less susceptible *Dendraster* the occurrence of a few exogastrulae—at most, 5–10 per cent (estimated)—may be attributed to the higher susceptibility to the dye of a few embryos or perhaps to incidental conditions, such as aggregation.

Although the ability to decolorize the dye in low concentrations is somewhat greater in daylight at the higher temperature, it is also to be noted that, with increase in concentration, even with the relatively low concentrations used in these experiments, the dye becomes more toxic in daylight lots than in those in darkness. With the concentrations used, this difference is less in the generally less susceptible *Dendraster* than in *Strongylocentrotus*. It is possible that the higher metabolism at the higher temperature renders the animals somewhat more susceptible to concentrations above a certain range. However, the differences at the two temperatures are greater than would be expected from the temperature differences alone, and, since they are distinguishable only in the higher concentrations and since in *Strongylocentrotus* the dye becomes much more highly lethal in daylight than in darkness, it is believed that a photodynamic effect is concerned.

The use of methylene blue, as well as of other reversible dyes, as an oxidation-reduction indicator on many organisms has shown that, with increasing intracellular concentration in daylight, the dye soon becomes a powerful irritant. In motile forms, e.g., many Protozoa, *Hydra*, planarians and other flatworms, annelids, developing fishes, and amphibians after motile stages are attained, there is an increase of motor activity, often becoming unco-ordinated, and eventually death. In darkness animals may become very deeply stained without evidence of irritation but show it at once on exposure to light.

Whether or to what extent a photodynamic effect may perhaps be a factor in the increase of respiration by methylene blue which has been observed by various investigators seems, so far as the writer has been able to learn, not to have been ascertained. Respiratory determinations on the same material in the same dye concentrations in daylight and in darkness would be of interest in this connection.

If photodynamic effects did occur in the daylight lots of the two echinoderms, it appears that the differential inhibitions by methylene blue and those which may be attributed to photodynamic effects

show the same general relation to the very similar gradient patterns of the two species as do the developmental modifications produced by a very considerable number of other external inhibiting agents (Child, 1941, pp. 129–39 and literature cited there; Rulon, 1941, 1948, 1950*a*, *b*).

It is to be noted, however, that the question of photodynamic effects in the echinoderm material is incidental to the chief purpose of the paper, which is to show that developmental modifications by methylene blue have the same differential character as those produced by many other agents. Moreover, the daylight lots of *Dendraster* and of *Strongylocentrotus* are not directly comparable. The daylight *Dendraster* material developed at temperatures 3°–4° C. higher than the other form and in the daylight of July and August, much stronger than the daylight of January and February, to which *Strongylocentrotus* was exposed; yet the latter was more susceptible. Also, *Dendraster* was exposed to daylight after 24 hours at the low temperature in darkness and *Strongylocentrotus* from early cleavage on. The two series were merely incidental attempts to determine whether evidence of a photodynamic effect might appear and whether it might play a part in developmental modification. The few data mentioned in this paper do little more than raise the question of the occurrence of a photodynamic effect and of its possible significance, not only as regards developmental modification but as regards metabolism. They have been noted chiefly as perhaps suggesting possibilities for future work. Obviously, for definite conclusions, adequate control of both temperature and light intensity are essential, and material from single males and females should be used in lots to be compared in light and darkness.

Developmental modifications produced by different agents may differ in certain respects, but it is not yet demonstrated that such differences are specific for a particular agent. Considerable difference may result from differences in the rapidity with which a particular agent becomes inhibitory to a certain degree. In general, these modifications indicate that, even after more or less differentiation has occurred, quantitative differentials without specific relations to any particular agent may still be extremely important in development. Differential modifications produced by differences in temperature alone are examples of such modifications (Child, 1950).

## SUMMARY

1. Methylene blue retards and modifies differentially the larval development of the echinoderms, *D. excentricus* and *S. purpuratus*, both in darkness at low temperature and in diffuse daylight at somewhat higher temperature. The differential modifications show the same general relations to the gradient pattern in both organisms as do those produced by many other external agents. In differential inhibition by the dye the regional differences characteristic of polarity and ventrodorsality in development decrease and are obliterated; in differential acclimation and differential recovery from dye effects, the regional differences become exaggerated.

2. In the lower dye concentrations used, the ectoderm is capable of decolorizing and apparently destroying the intracellular dye after some primary staining or apparently as rapidly as the dye enters. In gastrula and later stages, the entoderm and mesenchyme stain much more deeply and show much less ability to rid themselves of the dye. They become more inhibited in further development than does the ectoderm, and, with

increasing dye concentrations, the entoderm undergoes partial or complete dissociation, progressing from the anterior end, while the ectoderm may remain intact and undergo further development. On return to water, intracellular decoloration occurs more rapidly.

3. In the lower dye concentrations and with gradual intracellular decoloration, the modifications indicating differential recovery and probably in some cases differential acclimation occur. In higher concentrations with less decoloration, they become less extreme and less frequent. In *Dendraster* these secondary modifications occur to a much greater degree and in a wider range of concentrations than in the much more susceptible *Strongylocentrotus*.

4. The ability to decolorize the intracellular dye appears to be somewhat greater at the higher temperature in daylight in very low dye concentrations, and the secondary modifications occur more frequently and to a greater degree than in darkness at the lower temperature. With increasing concentration above a certain range, there is an increase in toxicity and in the lethal effect of the dye to a much greater degree than in darkness at the lower temperature. This is believed to be partly due to photodynamic effect. It is more extreme in *Strongylocentrotus* than in *Dendraster*, and in both forms shows the same relations to the gradient pattern as do other differential inhibitions by methylene blue and other agents. Whether it involves an increase in oxygen uptake is apparently not known.

## LITERATURE CITED


BARRON, E. S. G. 1929. Studies on blood cell metabolism. III. The effect of methylene blue on the oxygen consumption of the eggs of the sea urchin. The mechanism of the action of methylene blue on living cells. Jour. Biol. Chem., **81**: 445–57.

BARRON, E. S. G., and HOFFMAN, L. A. 1930. The catalytic effect of dyes on the oxygen consumption of living cells. Jour. Gen. Physiol., **13**:483–94.

BROOKS, MATILDA M. 1941. Differences in rates of $O_2$ consumption and of $CO_2$ production in fertilized and unfertilized *Arabacia* eggs. Proc. Amer. Physiol. Soc., pp. 39–40.

———. 1943. Methylene blue, potassium cyanide, and carbon monoxide as indicators for studying the oxidation-reduction potentials of developing marine eggs. Biol. Bull., **84**:164–77.

CHILD, C. M. 1940. Lithium and echinoderm exogastrulation. Physiol. Zoöl., **13**:4–42.

———. 1941. Patterns and problems of development. Chicago: University of Chicago Press.

———. 1948. Exogastrulation by sodium azide and other inhibiting conditions in *Strongylocentrotus purpuratus*. Jour. Exper. Zoöl., **107**:1–38.

———. 1950. Differential modification of sand-dollar development in relation to temperature. Physiol. Zoöl., **23**:140–68.

CLOWES, G. H. A., and KRAHL, M. E. 1936. Studies on cell metabolism and cell division. I. Jour. Gen. Physiol., **20**:145–71.

RULON, O. 1941*a*. The alteration of developmental pattern in the sand dollar by pilocarpine. Physiol. Zoöl., **14**:461–69.

———. 1941*b*. Modification of development in the sand dollar by NaCNS and Ca-free sea water. *Ibid.*, pp. 305–15.

———. 1948. The experimental modification of developmental patterns in *Arbacia* with malonic acid. *Ibid.*, **21**:100–105.

———. 1950*a*. The modification of developmental patterns in the sand dollar with sodium azide. *Ibid.*, **23**:236–47.

———. 1950*b*. The modification of developmental patterns in the sand dollar by thiourea. *Ibid.*, pp. 248–57.

SHAPIRO, H. 1948. Diminution of rate of cell division by reversible dyes coincident with enhancement of oxidations. Physiol. Zoöl., **21**:218–24.

# EXPERIMENTAL STUDIES ON THE EFFECT OF EXTERNAL AGENCIES ON THE SEXUALITY OF A MARINE COPEPOD[1]

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THE present study was carried on from 1935 to 1945 in the Zoölogical Institute of the Kyoto University under the direction of Professor T. Komai. A part of the results has been published in Japanese (1939, 1941*a*, *b*, 1948). In the present paper I intend to present the essentials of my findings, together with a general consideration on the problem of the sex determination in the copepod.

Ruttner (1930), Harada (1935), Kikuchi, Enoda, and Tateno (1942), and Mori (1945) give records on the sex ratio under natural conditions in several marine and fresh-water copepod forms. It has been found in both Diaptomidae and Cyclopidae that the number of males generally exceeds that of females more often in tropical lakes than in the lakes in the temperate zones. The sex ratio also shows seasonal changes; the number of males decreases in summer in *Diaptomus birlai* found in Formosa, in *D. gracilis* found in Lake Runz, and in *Eudiaptomus japonicus* found in Lake Biwa, whereas the number of males increases in *D. pacificus*, occurring in Lake Kizaki, and in *Mecocyclops taihokuensis*, occurring in Formosan lakes. Thus both the sex ratio and its seasonal changes vary in different species. But, so far as I know, no study on the sexual differentiation of copepods has been performed.

In the present study I have endeavored to analyze the mechanism of sexual differentiation. By adding certain chemicals to the culture medium in various concentrations and by transferring to this medium various stages of larvae, I have succeeded in changing the ordinary sex ratio of this copepod rather significantly. Thus I have been able to ascertain that the sexuality of this material is influenced considerably by environmental factors. I have also found that the sexuality is a function of the velocity of development in a certain larval stage.

The material was *Tigriopus japonicus* Mori, a marine copepod found commonly in tide pools in the warmer parts of Japan (Fig. 1). It is very hardy and can stand a great diversity of environmental conditions, so that it is not difficult to rear the animals in finger bowls. This genus propagates bisexually, no parthenogenesis being known.

The number of stages passed by this copepod in development from birth to maturity is 12—6 nauplius and 6 copepodid stages—the sixth copepodid stage being the adult one. The time from one ecdysis to another is nearly the same in all the stages under the same environmental conditions. The stages can be distinguished by peculiarities in external structure.

This copepod can live in fresh water for 2 months; also it can stand sea water saturated with table salt; but ordinary

[1] I wish to express my hearty gratitude to Professor T. Komai for his kind guidance and encouragement, given throughout the long course of my work. I wish also to acknowledge my great indebtedness to the Iwatare Shogakukai, whose financial aid has made this work possible. My thanks are also due to Dr. C. M. Child and Dr. W. C. Allee for valuable advice in publishing this paper.

sea water or sea water diluted with a small quantity of fresh water is the best culture medium.

The copepod can live at temperatures ranging from 0° to 30° C., and most of them can stand 34°. However, the optimum temperature for development is

## Method of Investigation

To examine the effect of chemicals on sexuality, the nauplii of the same brood were divided as evenly as possible into two groups, and, of these, one was reared in an artificial medium, while the other was kept in ordinary sea water as con-

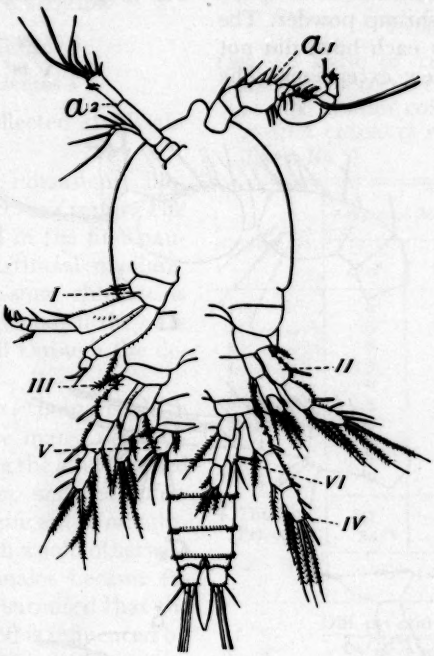


FIG. 1.—*Tigriopus japonicus* Mori male. × 80. $a_1$, antennule; $a_2$, antenna; I–VI, first–sixth legs

23°–25°, and signs of harmful effect often appear at 26°.

Sexual difference in the adult stage is so marked that the sexes can be distinguished under a hand lens (Fig. 2). Sexual difference is first recognized in the fourth copepodid stage, though rather indistinctly. In the next stage, copepodid V, this becomes distinct. In nature there is a tendency for males to be more abundant than females in spring and summer, and the reverse is the case in autumn and winter.

trol. After maturity the sex ratios of the two groups were compared. Care was taken to develop all the larvae to the adult, and, if even a single larva was lost during the course of the experiment, the entire culture was discarded.

Special care was paid to the following points: (1) The culture medium must be such as to insure the healthy and normal development of the control; (2) the medium must not change its quantity all through the experiment (100 cc. of medium in glass finger bowls was always

used). All the experiments except the temperature experiment were carried out at 23°, which is within the range of optimum temperature for the life of this copepod. The medium was renewed every day and was used after being warmed to 23°. The larvae were reared in finger bowls of hard glass and were fed with a proper quantity of shrimp powder. The number of larvae in each bowl did not exceed 20, with a few exceptions. The adults before oviposition were fed with sufficient food for several days at 23°.

To examine the size of the larvae in different stages, measurements were taken on the carapace of exuviae. For the statistical treatment of data, if the following relation held, the obtained value was judged as significant:

$$\frac{p_1 - p_2}{\sqrt{m_1^2 + m_2^2}} > 3 ,$$

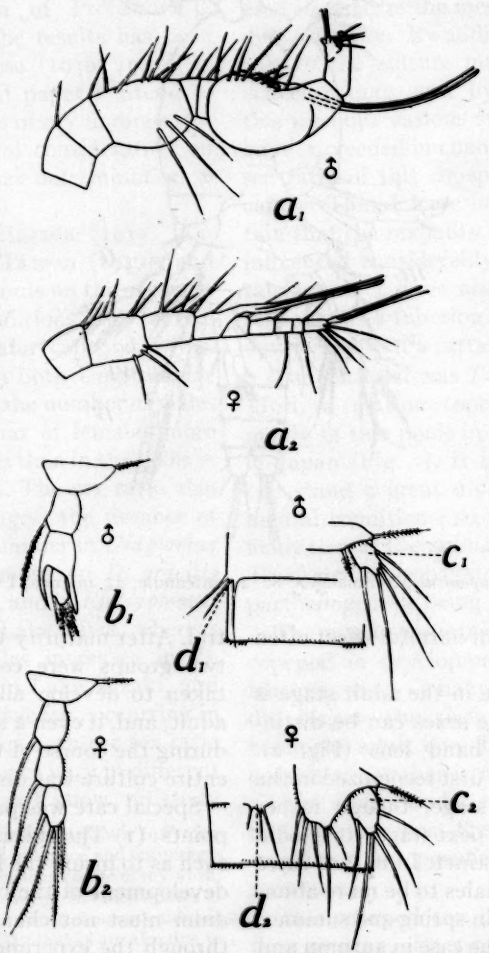


FIG. 2.—Sexual difference in antennule (*a*), endopodite of second leg (*b*), fifth leg (*c*), and sixth leg (*d*)

where $p_1$ is the percentage of females in culture in artificial medium; $p_2$ the percentage of females in the control; $m_1$ the standard error for $p_1$; and $m_2$ the standard error for $p_2$.

## The Effect of Potassium Chlorate and Potassium Chloride

### Potassium Chlorate

#### Experimental Series A

The material was collected at Misaki and Shimoda.

*Experiment No. 1.*—Potassium chlorate, 0.15 gm. per 100 cc. sea water. The larvae were transferred in the first nauplius stage into the artificial medium. This quantity of potassium chlorate is almost lethal to the copepods if they are kept in this medium all through the development.

As shown in Table 1, compared with the control, there were many more females than males among the adults raised in the artificial medium, and the difference is statistically significant. Undoubtedly, many larvae which would otherwise have developed into males became females. Thus it may be surmised that the sexuality of this copepod is influenced by environmental conditions, at least to some extent. This assertion has been substantiated by further experiments, to be mentioned later.

The development in the medium was retarded to such an extent that the time when they became copepodid I was delayed in many cases. The treated larvae had a shorter body than the control from copepodid I stage on, as shown in Table 2. Although no accurate measurement of body-length of the nauplii was taken, most of the treated larvae were decidedly smaller than the control. As may be seen in this table, the males, especially those in the control, usually had greater body-length than females in copepodid IV stage.

*Experiment No. 2.*—Potassium chlorate, 0.05 gm. per 100 cc. sea water (Table 3). The quantity of potassium chlorate added to sea water was next reduced to 0.05 gm. The effect of the chemical on the sex ratio disappeared. The development, however, was somewhat accelerated, and the copepodid I stage came sooner than in the control. In the later stages the rate of development decreased; the body-length increased, as shown in Table 4.

*Experiment No. 3.*—Potassium chlorate, 0.03 gm. + 100 cc. sea water (Table 3). The quantity of potassium chlorate was reduced further to 0.03 gm. In this medium a tendency suggesting increase of males appeared, though the difference in sex ratio from the control is statistically insignificant. The development was accelerated up to the early copepodid

TABLE 1

THE SEX RATIO OF THE COPEPOD, *Tigriopus japonicus* CULTURED FROM NAUPLIUS STAGE I IN THE MEDIUM CONTAINING 0.15 GM. POTASSIUM CHLORATE PER 100 CC. SEA WATER (EXPT. No. 1)

| Culture No. | Artificial Medium | | Control | |
|---|---|---|---|---|
| | ♂♂ | ♀♀ | ♂♂ | ♀♀ |
| 1 | 6 | 6 | 9 | 3 |
| 2 | 9 | 7 | 14 | 2 |
| 3 | 6 | 4 | 6 | 4 |
| 4 | 5 | 10 | 13 | 2 |
| 5 | 0 | 14 | 4 | 10 |
| 6 | 5 | 5 | 7 | 3 |
| 7 | 5 | 9 | 11 | 3 |
| 8 | 4 | 11 | 6 | 9 |
| 9 | 1 | 15 | 8 | 8 |
| 10 | 6 | 10 | 10 | 6 |
| Total | 47 | 91 | 88 | 50 |
| Per cent. | 34.1 | 65.9 | 63.8 | 36.2 |
| | $m_1 = 4.0$ | | $m_2 = 4.1$ | |

$$\frac{\text{Diff. per cent ♀♀}}{\sqrt{m_1^2 + m_2^2}} = 5.3 .$$

## TABLE 2

THE MEAN BODY-LENGTH (MM.) OF COPEPODID STAGES I–V CULTURED FROM NAUPLIUS STAGE I IN THE MEDIUM CONTAINING 0.15 GM. POTASSIUM CHLORATE PER 100 CC. SEA WATER (A) COMPARED WITH THE CONTROL (B) (EXPT. NO. 1)

| CULTURE NO. | STAGE | | | | | | |
|---|---|---|---|---|---|---|---|
| | I | II | III | IV | | V | |
| | | | | ♂ | ♀ | ♂ | ♀ |
| 4 A | 0.289 | 0.344 | 0.426 | 0.516 | 0.511 | 0.609 | 0.621 |
| 4 B | 0.311 | 0.366 | 0.449 | 0.532 | 0.517 | 0.650 | 0.600 |
| 7 A | 0.299 | 0.355 | 0.435 | 0.528 | 0.526 | 0.670 | 0.663 |
| 7 B | 0.309 | 0.372 | 0.447 | 0.551 | 0.548 | 0.672 | 0.649 |
| 8 A | 0.285 | 0.345 | 0.433 | 0.521 | 0.530 | 0.674 | 0.666 |
| 8 B | 0.301 | 0.364 | 0.452 | 0.603 | 0.538 | 0.679 | 0.693 |

## TABLE 3

THE SEX RATIO IN PER CENT OF THE COPEPOD *Tigriopus japonicus* CULTURED IN SEA WATER PLUS POTASSIUM CHLORATE AT 23°

| EXPT. SERIES NO. | REPLICATIONS | STARTING STAGE | GM. $KClO_3$ PER 100 CC. SEA WATER | ARTIFICIAL MEDIUM | | | CONTROL | | | (DIFF. PER CENT ♀♀)/$(\sqrt{m_1^2+m_2^2})$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | ♂♂ | ♀♀ | $m_1$ | ♂♂ | ♀♀ | $m_2$ | |
| A | 1 | 10 | Nauplius I | 0.15 | 34.1 | 65.9 | 4.0 | 63.8 | 36.2 | 4.1 | +5.3 |
| | 2 | 10 | Nauplius I | 0.05 | 59.1 | 40.9 | 4.2 | 62.8 | 37.2 | 4.1 | +0.6 |
| | 3 | 12 | Nauplius I | 0.03 | 71.3 | 28.7 | 3.4 | 59.8 | 40.2 | 3.7 | −2.3 |
| | 4 | 6 | Nauplius III | 0.15 | 35.1 | 64.9 | 4.8 | 68.0 | 32.0 | 4.6 | +5.0 |
| | 5 | 6 | Nauplius V | 0.15 | 57.0 | 43.0 | 4.9 | 74.0 | 26.0 | 4.4 | +2.6 |
| | 6 | 6 | Earlier period, first half, nauplius VI | 0.15 | 58.9 | 41.1 | 5.4 | 62.2 | 37.8 | 5.1 | +0.4 |
| B | 7 | 6 | Later period, first half, nauplius VI | 0.15 | 81.3 | 18.7 | 4.1 | 79.1 | 20.9 | 4.3 | −0.4 |
| | 8 | 12 | Later period, second half, nauplius VI | 0.15 | 71.3 | 28.7 | 3.3 | 55.1 | 44.9 | 3.7 | −3.3 |
| | 9 | 10 | Copepodid I | 0.15 | 63.5 | 36.5 | 3.9 | 62.9 | 37.1 | 3.9 | −0.1 |
| | 10 | 16 | Nauplius III | 0.15 | 66.8 | 33.2 | 2.8 | 77.0 | 23.0 | 2.5 | +2.7 |
| | 11 | 10 | Nauplius V | 0.15 | 48.3 | 51.7 | 3.7 | 72.8 | 27.2 | 3.3 | +4.9 |
| | 12 | 10 | First half, nauplius VI | 0.15 | 42.2 | 57.8 | 3.7 | 65.0 | 35.0 | 3.6 | +4.4 |
| C | 13 | 15 | Second half, nauplius VI | 0.15 | 54.0 | 46.0 | 3.1 | 68.3 | 31.7 | 2.9 | +3.4 |
| | 14 | 12 | Copepodid I | 0.15 | 62.6 | 37.4 | 3.3 | 63.0 | 37.0 | 3.3 | +0.1 |
| | 15 | 10 | Copepodid I | 0.3 | 56.3 | 43.7 | 3.8 | 56.9 | 43.1 | 3.8 | +0.1 |
| | 16 | 10 | Second half, nauplius VI | 0.3 | 63.6 | 36.4 | 3.7 | 67.3 | 32.7 | 3.7 | +0.7 |

stages, but after that it was retarded. The copepodids had longer bodies than the control.

In the series of experiments presented above, the larvae were reared from the first nauplius stage in the medium containing 0.03–0.15 gm. of potassium chlorate per 100 cc. sea water. The use of 0.15 gm. potassium chlorate induced a distinct tendency toward the increase of females. This tendency disappeared entirely at 0.05 gm.

TABLE 4

THE MEAN BODY-LENGTH (MM.) OF COPEPODID STAGES I–V CULTURED FROM NAUPLIUS STAGE I IN THE MEDIUM CONTAINING 0.05 GM. POTASSIUM CHLORATE PER 100 CC. SEA WATER (A) COMPARED WITH THE CONTROL (B) (EXPT. NO. 2)

| Culture No. | | Stage I | II | III | IV ♂ | IV ♀ | V ♂ | V ♀ |
|---|---|---|---|---|---|---|---|---|
| 6 A | 0.05 per cent $KClO_3$ | 0.308 | 0.379 | 0.450 | 0.537 | 0.540 | 0.667 | 0.646 |
| 6 B | Control | 0.305 | 0.362 | 0.440 | 0.516 | 0.527 | 0.642 | 0.629 |
| 10 A | 0.05 per cent $KClO_3$ | 0.289 | 0.362 | 0.437 | 0.540 | 0.532 | 0.644 | 0.651 |
| 10 B | Control | 0.284 | 0.360 | 0.428 | 0.522 | 0.513 | 0.637 | 0.639 |

EXPERIMENTAL SERIES B. EXPERIMENTS ON LARVAE OF DIFFERENT STAGES REARED IN ARTIFICIAL MEDIUM (TABLE 3)

To determine the critical developmental stage for the determination of sex in this material, larvae of different stages were transferred to the artificial medium and reared to the adult stage. The medium contained 0.15 gm. of potassium chlorate per 100 cc. sea water.

*Experiment No. 4. The III nauplius stage larvae.*—A distinct tendency toward the increase of females is perceived, as in the preceding experiment. The first copepodid stage came later than in the control.

*Experiment No. 5. The V nauplius stage larvae.*—The tendency toward increase of females is perceived in this case also, although this is not so marked as in the preceding case. The copepodid stage I came later than in the control.

*Experiment No. 6. Larvae in the earlier period of the first half of nauplius VI.*—The tendency toward increase of females, which was apparent in the preceding experiments, is no longer to be found. The first copepodid stage was reached at nearly the same time as with the control.

*Experiment No. 7. Larvae in the later period of the first half of nauplius VI.*—The tendency which was apparent in experiments 1 and 2 can hardly be perceived here. Development to copepodid I was normal; but it was retarded after that, and the adult stage came later than with the control.

*Experiment No. 8. Larvae in the later period of the second half of nauplius VI.*—The larvae were divided into two groups several hours before they arrived at stage copepodid I, and one group was reared in the artificial medium. A tendency suggesting the increase of males in the treated group appeared. The time at which they became copepodid I did not differ from that in the control; but the

time when they became adult was somewhat delayed.

*Experiment No. 9. Copepodid I stage larvae.*—No tendency toward the increase of males or females was seen in this case.

In the above nine experiments of series A and B, the larvae in various stages of development were placed in sea water with potassium chlorate added, usually at the strength of $KClO_3$, 0.15 gm. + 100 cc. ordinary sea water, and reared to the adult. When the larvae of the nauplius stages I to III were used, a remarkable increase of females resulted. This tendency became less pronounced when the larvae were in slightly later stages; and it disappeared entirely when the early nauplius VI larvae were transferred to the medium. In the later nauplius VI stage, however, a new tendency suggesting the increase of males set in, to disappear again in the case of copepodid stage I. Throughout the experiments no intersex was found, at least so far as the external characters were concerned.

EXPERIMENTAL SERIES C

These experiments were carried out at Seto Marine Biological Station on the material collected in its vicinity. This material was slightly different in its physiological state from that of the same species collected at Misaki or Shimoda and used in the foregoing experiments. For example, development of the Seto material was faster than that of the Misaki or Shimoda material, and it took only 6–8 days (in the former, 8–11 days) from birth to maturity at 23°. This is probably due to the fact that the material was fresh from the sea and was more vigorous than the Misaki and Shimoda material. I carried out the same kind of experiments on this material as those just stated. The results are practically identical with those obtained on the Misaki or Shimoda material mentioned above, as shown in Table 3.

The medium used was potassium chlorate, 0.15 gm. + 100 cc. sea water. Larvae in different stages were transferred to this medium.

*Experiment No. 10. Nauplius III stage larvae.*—Some tendency toward the increase of females was perceived, but it was not so marked as in the preceding experiments. The time when the larvae became copepodid was considerably later than in the control.

*Experiment No. 11. Nauplius V stage larvae.*—The same tendency appeared. The copepodid stage came later than in the control, as in the preceding experiment.

*Experiment No. 12. Larvae in the first half of nauplius VI stage.*—The same tendency appeared. Attainment of copepodid I stage was delayed. (The experiments on the material from Misaki and Shimoda did not show this difference.)

*Experiment No. 13. Larvae in the latter half of nauplius VI stage.*—Special care was taken not to transfer the larvae to the artificial medium just before molting. The same tendency was perceived. The copepodid I stage was attained nearly simultaneously with the control.

*Experiment No. 14. Copepodid I stage larvae.*—No difference from the control was apparent.

The medium used was potassium chlorate, 0.3 gm. + 100 cc. sea water. The quantity of potassium chlorate was increased to 0.3 gm. for 100 cc. sea water.

*Experiment No. 15. Copepodid I stage larvae.*—No effect.

*Experiment No. 16. Larvae in the second half of nauplius VI stage.*—No effect on sexuality. Attainment of copepodid I stage, however, was considerably later than in the control.

In experiments 10–14 the larvae in various developmental stages were trans-

ferred from sea water to the medium, $KClO_3$, 0.15 gm. + 100 cc. sea water. Those transferred to the medium at the nauplius stage III showed some tendency toward the increase of females among the adults. This tendency was more marked in the lot reared in the medium from nauplius stage V or from the earlier part of stage VI. The same tendency was also recognizable, though less definitely, in the lot brought into the medium in the latter part of nauplius stage VI. But, from copepodid I on, no such tendency is to be seen.

In experiment No. 15 the larvae were reared from the copepodid stage I in the medium containing 0.3 gm. $KClO_3$ per 100 cc. sea water. This concentration of $KClO_3$ failed to influence the sexuality of the copepod, probably because the copepodid stage I was too late in development to be sexually labile. Even the latter part of nauplius VI stage was too advanced to bring about such a change in sexuality, judging from the negative result obtained in experiment No. 16. Throughout these experiments no intersex was found, so far as the external characters were concerned.

### POTASSIUM CHLORIDE (EXPERIMENTAL SERIES D)

In the experiments described in the preceding section the potassium chlorate added to the sea water was found to influence the sexuality of the copepod. Presumably the effect was due to the increase of the potassium ion in the sea water. Therefore, potassium chloride was tried for the same purpose.

*Experiment No. 17.*—The nauplius VI larvae were brought into the medium containing 0.15 gm. potassium chloride per 100 cc. sea water. The rate of development in the latter part of nauplius VI stage decreased, and a clear tendency toward the increase of females appeared.

*Experiment No. 18.*—The larvae in copepodid I stage were placed in the medium containing 0.15 gm. potassium chloride per 100 cc. sea water. No change in sexuality appeared.

The above experiments show that potassium chloride added to sea water also influences the sexuality of the copepod. However, when the larvae were in copepodid stage I, the sex could no longer be altered.

In all the experiments presented above, the larvae in various stages of development were brought into a medium containing various concentrations of potassium chlorate or potassium chloride. Thus I have been able to ascertain that the sexuality of this copepod is influenced by the medium in which it develops. The sex ratio can be shifted toward the increase of females or males by adding the proper quantity of these chemicals to the sea water. At the same time, the rate of development is somewhat altered. Let us see how these changes are interrelated.

The findings set forth above seem to favor the view that the sex of the copepod is determined rather abruptly at the outset of the first copepodid stage. Before this stage the larva is still sexually undifferentiated. Its physiological state in the latter part of nauplius VI stage seems to play a decisive role in the determination of sex. An agent retarding development in this critical stage induces a higher percentage of females. The experiments seem to justify this generalization. The only exception is experiment No. 16, in which the larvae were in the medium containing 0.3 gm. $KClO_3$ per 100 cc. sea water. Here no obvious effect on sexuality appeared, in spite of the considerable decrease in developmental velocity in the latter part of nauplius VI stage. In experiment No. 10, in which nauplius III larvae were in the medium containing 0.15 gm. $KClO_3$ per 100 cc. sea water, the

increase of females was not so pronounced as in other experiments, although the developmental velocity was most markedly decreased. In both these cases the material was from the sea off Seto and was found to be more sensitive to the chemical than that from Misaki and Shimoda. Moreover, in these experiments the quantity of the chemical added to the medium was very near the extreme that was endurable for the copepod. It is possible that such a quantity of the chemical would upset the above relation; hence there was no apparent influence on sexuality in experiment No. 16.

The effect on sexuality of potassium chloride added to sea water was similar to that of potassium chlorate. Probably the influence of these chemicals is mainly due to the increase of the potassium ion in the sea water, although the $ClO_3$ ion may be of some importance in the case of potassium chlorate. The role which the potassium ion plays in sex control is emphasized by C. Herbst (1935) for *Bonellia*, by K. Tzonis (1938) for *Dinophilus*, and by M. Hartmann and G. von Lewinski (1938) for *Ophryotrocha*. These authors have carried out a series of experiments with different quantities of the potassium ion in sea water, but I shall not enter into this question at present.

## The Effect of Chloretone, Chloral Hydrate, and Diiodotyrosin

To see whether the general rule stated above holds for other chemicals producing the same kind of effect on development, chloretone, chloral hydrate, and diiodotyrosin were used. Chloretone and chloral hydrate were chosen as reagents which retard development, while diiodotyrosin was used for its accelerating influence on development. The experiments were focused on the late nauplius VI stage, which was found to be the critical period for the determination of sex in this material in the preceding experiments.

As a preliminary experiment (Expt. No. 19) the larvae (nauplius stage VI) were left for 1 day at 23° in the sea water in which the chemicals were added in different concentrations, as shown in Table 5.

TABLE 5

THE CONCENTRATION OF CHLORETONE AND CHLORAL HYDRATE, SHOWING INJURIOUS EFFECTS ON NAUPLIUS VI STAGE LARVAE KEPT FOR 1 DAY

| Chemicals | Concentration Where They Looked Enfeebled (Gm. per 100 Cc. Sea Water) | Lethal Concentration (Gm. per 100 Cc. Sea Water) |
|---|---|---|
| Chloretone..... | 0.005 | 0.02 |
| Chloral hydrate..... | 0.01 | 0.05 |

### CHLORETONE

Chloretone 0.005 gm. was added to 100 cc. sea water. The larvae left in this medium for 1 day showed some toxic effect.

*Experiment No. 20.*—VI nauplius stage larvae were placed in the medium containing 0.005 gm. chloretone per 100 cc. sea water and left for 1 day. A feminizing tendency appeared (see Table 6); and the time when they reached the copepodid I stage was delayed as compared with that of the control.

*Experiment No. 21.*—I copepodid stage larvae were brought into the same strength of medium and were similarly left for 1 day. As shown in Table 6, neither feminizing nor masculinizing tendency appeared.

### CHLORAL HYDRATE

*Experiment No. 22.*—Chloral hydrate 0.01 gm. was added to 100 cc. sea water. In this medium the larvae showed toxic

effects in 1 day, as in the case of chloretone, and a feminizing tendency appeared in nauplius larvae stage VI. The copepodid stage was delayed, compared with that of the control.

*Experiment No. 23.*—Copepodid I stage larvae were brought into the same medium for 1 day. There was no feminizing or masculinizing tendency.

Thus both chloretone and chloral hydrate retarded development in the latter part of nauplius VI stage and induced feminization, while diiodotyrosin accelerated it and induced masculinization. When they were brought into these media in copepodid I stage, the sexuality was no longer altered. Throughout these experiments no intersex appeared, so far as the external characters were concerned.

TABLE 6

THE SEX RATIO IN PER CENT OF THE COPEPOD *Tigriopus japonicus* CULTURED IN SEA WATER PLUS POTASSIUM CHLORIDE, CHLORETONE, CHLORAL HYDRATE, OR JORGON AT 23°

(SERIES D)

| Expt. Series No. | Replications | Starting Stage | Chemical Dissolved in 100 Cc. Sea Water (In Gm.) | Artificial Medium | | | Control | | | $\frac{(p_1-p_2)}{(\sqrt{m_1^2+m_2^2})}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | ♂♂ Per Cent | ♀♀ Per Cent ($p_1$) | $m_1$ | ♂♂ Per Cent | ♀♀ Per Cent ($p_2$) | $m_2$ | |
| 17.... | 10 | Nauplius VI | Potassium chloride, 0.15 | 46.1 | 53.9 | 3.7 | 62.2 | 37.8 | 3.6 | +3.1 |
| 18.... | 10 | Copepodid I | Potassium chloride, 0.15 | 65.6 | 34.4 | 3.5 | 66.7 | 33.3 | 3.5 | +0.2 |
| 20.... | 10 | Nauplius VI | Chloretone, 0.005 | 46.6 | 59.4 | 3.7 | 62.2 | 37.8 | 3.6 | +4.2 |
| 21.... | 10 | Copepodid I | Chloretone, 0.005 | 66.7 | 33.3 | 3.5 | 66.1 | 33.9 | 3.5 | −0.1 |
| 22.... | 10 | Nauplius VI | Chloral hydrate, 0.01 | 36.7 | 63.3 | 3.6 | 58.3 | 41.7 | 3.7 | +4.2 |
| 23.... | 10 | Copepodid I | Chloral hydrate, 0.01 | 60.0 | 40.0 | 3.7 | 59.4 | 40.6 | 3.7 | −0.1 |
| 24.... | 15 | Nauplius VI | Jorgon, 0.01 | 70.7 | 29.3 | 2.8 | 56.3 | 43.7 | 3.0 | −3.4 |
| 25.... | 10 | Copepodid I | Jorgon, 0.01 | 64.4 | 35.6 | 3.6 | 65.6 | 34.4 | 3.5 | +0.2 |

### DIIODOTYROSIN

Diiodotyrosin (Jorgon) was used for accelerating development. It contains 58 per cent of iodine and is soluble in sea water. Jorgon 0.1 gm. added to 100 cc. sea water was found to be rather too strong for the normal development of the larvae.

*Experiment No. 24.*—VI nauplius stage larvae brought into the medium, 0.01 gm. Jorgon + 100 cc. sea water, and left for 1 day showed a masculinizing tendency. The time when they reached the copepodid I stage was hastened.

*Experiment No. 25.*—Copepodid I stage larvae similarly treated with the Jorgon medium showed neither masculinizing nor feminizing tendency.

In these experiments chemicals known to decrease or to increase the developmental velocity were used. It has become evident that they exert a rather strong influence on the differentiation of sex in this copepod. Thus the relation found in the preceding experiments holds for these chemicals also.

### THE EFFECT OF TEMPERATURE

EXPERIMENTAL SERIES E, TABLE 7

The findings on the effect of chemicals on development and sexuality induced the writer to perform another series of experiments of rearing the larvae under different temperatures. As already mentioned, 23° was confirmed as within the

optimum temperature for the life of this copepod, and all the experiments discussed so far were made at that temperature. However, 18° seems to be nearly as good, judging from the length of life of the copepod and also the number of eggs laid. For development, however, 23° is obviously better than 18°, the velocity at 23° is nearly one and a half times that at 18°. By using the same method as in the previous experiments, the sex ratios of the two lots reared at 23° and 18°, respectively, were compared.

*Experiment No. 26.*—Nauplius II stage larvae transferred to 18° sea water. A disproportionately large number of females was produced, as compared with those reared at 23°. One intersex individual appeared in this experiment. It had antennules intermediate between male and female, but the other parts of the body were almost those of a normal female, and it produced offspring. The copepodids reared at 18° had a longer body than those raised at 23°, as shown in Table 8. The same was apparently true for the nauplius VI stage larvae.

*Experiment No. 27.*—Nauplius VI stage larvae transferred to 18° water produced more females than those left at 23°.

TABLE 7

THE SEX RATIO IN PER CENT OF THE COPEPOD *Tigriopus japonicus* CULTURED UNDER DIFFERENT TEMPERATURES

| Expt. Series No. | Replications | Starting Stage | 18° ♂♂ (Per Cent) | 18° ♀♀ (Per Cent) ($p_1$) | 18° $m_1$ | 23° ♂♂ Per Cent | 23° ♀♀ Per Cent ($p_2$) | 23° $m_2$ | $(p_1-p_2)/(\sqrt{m_1^2+m_2^2})$ |
|---|---|---|---|---|---|---|---|---|---|
| E 26..... | 12 | Nauplius II | 17.8 | 82.2 | 2.8 | 72.3 | 27.7 | 3.3 | +12.7 |
| E 27..... | 10 | Nauplius VI | 52.8 | 47.2 | 3.7 | 70.6 | 29.4 | 3.4 | + 3.5 |
| E 28..... | 12 | Copepodid I | 34.0 | 66.0 | 3.7 | 32.1 | 67.9 | 3.7 | − 0.4 |
| E 29..... | 12 | Copepodid I | 73.3 | 26.7 | 3.1 | 73.8 | 26.2 | 3.1 | + 0.1 |

TABLE 8

THE MEAN BODY-LENGTH (MM.) OF THE COPEPODIDS RAISED AT DIFFERENT TEMPERATURES FROM NAUPLIUS II STAGE

| Culture No. | Temp. | Stage I | Stage II | Stage III | Stage IV ♂ | Stage IV ♀ | Stage V ♂ | Stage V ♀ |
|---|---|---|---|---|---|---|---|---|
| 1 A..... | 18° | 0.331 | 0.409 | 0.505 | 0.604 | 0.618 | 0.755 | 0.764 |
| 1 B..... | 23° | 0.318 | 0.392 | 0.487 | 0.595 | 0.585 | 0.757 | 0.731 |
| 3 A..... | 18° | 0.341 | 0.411 | 0.495 | 0.617 | 0.623 | 0.767 | 0.785 |
| 3 B..... | 23° | 0.330 | 0.394 | 0.473 | 0.599 | 0.594 | 0.746 | 0.766 |
| 4 A..... | 18° | 0.307 | 0.367 | 0.461 | 0.567 | 0.575 | 0.698 | 0.693 |
| 4 B..... | 23° | 0.283 | 0.359 | 0.444 | 0.539 | ..... | 0.680 | ..... |
| 5 A..... | 18° | 0.301 | 0.372 | 0.469 | 0.590 | 0.601 | 0.736 | 0.754 |
| 5 B..... | 23° | 0.287 | 0.358 | 0.456 | 0.567 | 0.540 | 0.710 | 0.701 |

*Experiment No. 28.*—Copepodid I stage larvae transferred to 18° water produced no increase of males or females, as compared with the lot at 23°.

The above experiments show that, when the larvae were brought into 18° water in nauplius II or VI stages, a significant increase of females resulted as compared with the lots reared at 23°. The copepodid I stage was too late to yield the same result. From these findings we learn that the previously mentioned relations (see points *a* and *b* in the discussion below) hold good as to the temperature also.

## Discussion and Conclusions

All the experiments presented above with the five organic or inorganic substances and with temperature have yielded the following uniform results:

*a) The substances or conditions which accelerate development in the latter part of the nauplius VI stage induce masculinization, while those which decrease it induce feminization.*

*b) The sexuality of this copepod is determined rather abruptly at the outset of the copepodid stage I; before this the larvae are still in a neutral state.*

The experiments using chemicals have shown that sex could no longer be altered in the copepodid I stage. The temperature experiments also gave the same result. As stated above, the alteration of the velocity of development seems to be causally connected with the change in sexual ratio. At the same time, it is not impossible that the change in the viscosity of protoplasm or the change in degree of dispersion or in oxidation-reduction potential is more closely related to the sex differentiation of this copepod.

In this material the sexual differentiation of the gonad appears later than the external sexual characters, and there can be no possibility that the latter develops under the influence of the former. This is apparently the reason why practically no intersex was found in any experiments.

The results obtained by previous authors on the sex determination of various aquatic invertebrates are quite varied. For example, Shull and Ladoff treated the rotifer *Hydatina* with various substances, including ammonium salts, sodium hydroxide, beef extracts, and manure solution (1916), and the effect appeared in the generation of grandchildren. Brown and Banta (1932, 1935) reared daphnids at high temperature, and the effect appeared in the next generation. Furthermore, the mechanism of sex determination and differentiation is apparently different in different organisms. In some cases the sex is determined in an early stage, while in others it is changeable even after external sexual characters have appeared. Besides, there is apparently no definite correlation between metabolic rate and sexuality common to all sorts of animals. In rotifers, for instance, males are produced when the vitality of the strain is still high. The same is true for copepods. In *Cladocera*, on the contrary, males are produced when the vitality of the strain has diminished. According to Amemiya (1928), a tendency to feminization appears in the oyster when it is in vigorous condition. It seems to me that the reason for this discrepancy lies in the fact that the vitality of the material used in those experiments was concerned with that of the whole body of the organism, while the sexuality of the whole body does not necessarily coincide with the sexuality of its gonad. In any case it seems necessary that, in order to determine the causal relation of vitality and sexuality, individual cases should be critically examined.

As the cause of seasonal variation in the sex ratio of this copepod observed in

nature, selective mortality or selective fertilization has been assumed. The present study suggests that this variation is mainly due to the direct influence of temperature on the sexuality of the copepods.

## Summary

1. The marine copepod, *T. japonicus* Mori, is very easy to rear in finger bowls in the laboratory. The number of stages from birth to maturity is 12, namely, 6 nauplius and 6 copepodid stages, the sixth copepodid stage being the adult one. The sexual distinction appears first in the fourth copepodid stage. Throughout the experiments no intersex was found, with a single exception, at least so far as the external characters were concerned.

2. A series of experiments in which various concentrations of potassium chlorate or potassium chloride were added to culture media, into which various stages of the larvae were transferred, seems to have established the fact that (*a*) a chemical which increases the developmental velocity in the latter part of nauplius VI stage increases frequency of males, while that which decreases it increases frequency of females, and (*b*) the sex of this copepod is determined rather abruptly at the outset of the first copepodid stage. Up to this stage the larvae remain largely sexually neutral. The physiological state in the latter part of nauplius VI seems to play a decisive role in determining whether the larva will become a male or a female.

3. In order to determine whether these relations hold for other agents producing similar effects on development, chloretone and chloral hydrate were chosen as substances which bring about decrease of developmental velocity, and diiodotyrosin was used as a substance which increases it. The results of the experiments with these chemicals have shown that these substances also exert a significant influence on the determination of the sex of the copepod and that relations *a* and *b* hold good in these cases also.

4. This finding has induced the writer to carry out another series of experiments of rearing the larvae at different temperatures. It has been ascertained that relations *a* and *b*, mentioned above, hold for this case also.

5. The seasonal variation in sex ratio of copepods observed in nature is apparently due to the direct effect of temperature on sex determination.

## Literature Cited

Amemiya, I. 1928. A preliminary note on the sexuality of a dioecious oyster, *Ostrea gigas* Thunberg. Jap. Jour. Zoöl., 2:99–102.

Brown, L. A., and Banta, A. M. 1932. Sex control in *Cladocera*. VII. Male production in relation to temperature. Physiol. Zoöl., 5:218–29.

———. 1935. Control of sex in *Cladocera*. VIII. Food and crowding as factors in male production. Physiol. Zoöl., 8:138–55.

Harada, I. 1935. On the sex ratio of fresh-water copepods. Nihon-Gakujutsukyokai-Hokoku, 10: 728–30. (In Japanese.)

Hartmann, M., and Lewinski, G. von. 1938. Untersuchungen über die Geschlechtsbestimmung und Geschlechtsumwandlung von *Ophryotrocha puerilis*. II. Versuche über die Wirkung von Kalium, Magnesium und Kupfer. Zool. Jahrb., 58:551–74.

Herbst, C. 1935. Untersuchungen zur Bestimmung des Geschlechts. IV. Mitteilung. Die Abhängigkeit des Geschlechts vom Kaliumgehalt des umgebenden Mediums bei *Bonellia viridis*. Roux's Archiv, 132:576–99.

Kikuchi, K.; Enoda, Y.; and Tateno, F. 1942. Survey on the annual change of vertical distinction of plankton in Lake Biwa. Rikusuigaku Zassi, 12:63–72. (In Japanese.)

Mori, S. 1945. Annual change of plankton in the southern part of Lake Biwa during the years 1934–1937. Kyototeikokudaigaku Rigakubu Dobutsugakukyoshitsu. Otsu Rinkojikkensho Seiri-Seitaigaku Kenkyugyoseki, No. 53, pp. 1–28. (In Japanese.)

Ruttner, F. 1929. Das Plankton des Lunzer Untersees: Seine Verteilung in Raum und Zeit während

der Jahre 1908–1913. Internat. Rev. Hydrobiol., Leipzig, 23:1–138, 161–287.

SHULL, A. F., and LADOFF, S. 1916. Factors affecting male-production in *Hydatina*. Jour. Exper. Zoöl., 21:127–61.

TAKEDA, N. 1939. Sex determination and sex differentiation in the marine copepod, *Tigriopus japonicus* Mori. I. The influence of potassium chlorate (a preliminary note). Zoöl. Mag. Tokyo, 51:1–13. (In Japanese with English résumé.)

———. 1941*a*. Sex determination and sex differentiation in the marine copepod, *Tigriopus japonicus* Mori. I. The influence of potassium chlorate and potassium chloride. *Ibid.*, 53:22–31. (In Japanese with English résumé.)

———. 1941*b*. Sex determination and sex differentiation in the marine copepod, *Tigriopus japonicus* Mori. II. The influences of chloretone, chloral hydrate, and diiodotyrosin. *Ibid.*, pp. 158–70. (In Japanese with English résumé.)

———. 1948. Experimental studies on the external influence on the sexuality of the marine copepod *Tigriopus japonicus* Mori. III. Effect of temperature. Seibutsu, 3:12–21. (In Japanese with English résumé.)

TZONIS, K. 1938. Beeinflussung der Geschlechtsverhältnisse bei *Dinophilus apatris* Korsch. durch Aussenbedingungen. Zool. Jahrb., 58:539–50.

# OXYGEN UPTAKE OF INTACT EMBRYOS, THEIR HOMOGENATES, AND INTRACELLULAR CONSTITUENTS[1]

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As a result of the so-called "cell fractionation" and vital-staining techniques, considerable data are available relating to the basic nature of the various morphological constituents of living cells (Bensley and Hoerr, 1934*a*, *b*; Schneider, Claude, and Hogeboom, 1948). Many of these studies have dealt almost exclusively with vertebrate materials, particularly the liver, kidney, and brain. The enzymatic activities of fractions so isolated have been studied rather extensively, and a large amount of extremely interesting information concerning the localization and activity of intracellular enzymes has been obtained (Schneider, 1946). Inasmuch as the use of warm-blooded forms requires extremes in technique, such as refrigeration, low temperatures, etc., and since few, if any, data concerning these problems in invertebrates seem to exist, a "simple" cold-blooded invertebrate form was chosen for study. The embryo of the common grasshopper, *Melanoplus differentialis*, was selected, since considerable information concerning its morphology and physiology are already at hand (Bodine, 1950*b*). The fact that these embryos during their normal course of development at constant temperature (25° C.) go through a blocked or mitotically inactive state makes possible a rather critical study of their enzymatic activity and of the parts played by the various cell constituents during both mitotically active and blocked conditions. Embryos of known age and temperature history are readily dissected free from yolk and obtained in large numbers as more or less naked masses of embryonic cells. The present paper, therefore, has to do with a discussion of the oxygen uptake of intact embryos, their homogenates, and various intracellular constituents.

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## MATERIALS AND METHODS

Embryos were dissected free of yolk and suspended in a 0.90 per cent NaCl phosphate-buffered (pH 6.8) medium. Diapause and postdiapause embryos of

similar morphological appearance were used. The degree and extent of the diapause block in embryos were judged by the rates of lowered oxygen uptake, as well as by the developmental history of the eggs (Bodine, 1934). One hundred embryos per 1 ml. of medium were employed throughout all experiments, and to this extent all results are comparable. Oxygen determinations were carried out at 25° C. with standard Warburg manometers, with respiration flasks of 5-ml. capacity.

Homogenates were prepared according to the methods of Potter and Elvehjem (1936) at room temperatures, where they were kept until put into water baths at 25° C. No injurious effects of exposure to room temperatures for periods of 2–4 hours were detected. Centrifugation of homogenates was carried out in plastic tubes and with known forces of gravity, as indicated below in the discussion of the results of the experiments.

Further details of procedure were similar to those previously pointed out (Bodine, 1950*b*).

## RESULTS

### RELATIONS BETWEEN OXYGEN UPTAKE OF NORMAL AND BLOCKED INTACT EMBRYOS AND THEIR HOMOGENATES

The oxygen uptake of intact diapause (blocked) and postdiapause (active) embryos, as well as of homogenates made from them, was determined. No additional substrates were added, so that the enzyme activity studied was entirely confined to inherent enzyme-substrate systems. Typical results are graphically shown in Figure 1. An examination of this figure shows that the intact blocked or diapause embryos respire at a much lower rate than do the active, postdiapause ones. This marked lower rate of oxygen uptake of the diapause embryo has been repeatedly pointed out (Bodine, 1934) and perhaps represents a true basal rate for such a physiological state. The active or postdiapause embryo, on the other hand, has and maintains a much higher rate of oxygen uptake. Morphologically, these embryos are, to all intents and purposes, grossly similar in appearance and differ only in rates of oxygen uptake and mitotic activity of the cells (Bodine, 1934; Slifer, 1931). The oxygen uptake of

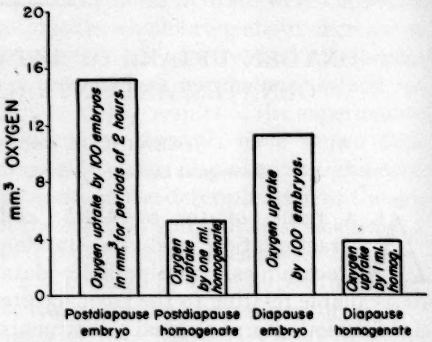



FIG. 1.—Shows oxygen uptake of postdiapause and diapause embryos and homogenates.

the respective homogenates is much reduced from that of the intact embryos (Fig. 1). Curiously, the relative difference in oxygen uptake of active and blocked homogenates is practically identical with that for the intact embryos (Table 1). The low oxygen uptake of the blocked embryo carries over to its homogenate, as do the higher values for the active one. The diapause or blocked state apparently seems like a deep-seated basic one which is closely associated with the intimate chemistry of the organism. If one considers the oxygen uptake of the intact embryo as 100 per cent, it is then readily seen from these results that approximately 65 per cent of this value is due to what one may term "structure," while 35 per cent is due to the basic chemical composition of the cells or or-

ganism. These relative percentage values seem to hold for both the active and the blocked states of the embryo (Fig. 1; Table 1).

and cytological studies were made of all precipitates (see Pl. I). Further details of procedures, etc., are indicated on charts and need no further explanation

TABLE 1

OXYGEN UPTAKE IN CUBIC MILLIMETERS BY 100 EMBRYOS (POSTDIAPAUSE) OR 1 ML. HOMOGENATE EQUIVALENT TO 100 EMBRYOS, IN 2 HOURS

(Shows Relations between Oxygen Uptake of Embryos and Homogenates)

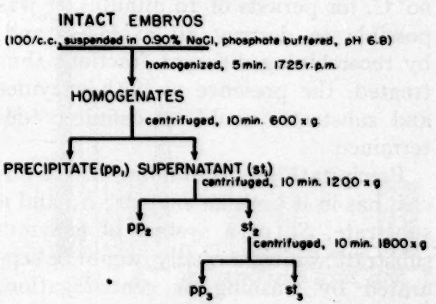
| Postdiapause Embryo | Postdiapause Homogenate | Diapause Embryo | Diapause Homogenate | P.D. Embryo/D. Embryo | P.D. Homogenate/D. Homogenate | P.D. Embryo/P.D. Homogenate | D. Embryo/D. Homogenate |
|---|---|---|---|---|---|---|---|
| 14.50 | 4.70 | 12.10 | 3.38 | 1.20 | 1.39 | 3.08 | 3.58 |
| 14.50 | 5.65 | 9.98 | 3.50 | 1.45 | 1.61 | 2.59 | 2.85 |
| 14.00 | 5.40 | 10.40 | 3.50 | 1.34 | 1.54 | 2.60 | 2.97 |
| 16.90 | 4.80 | 11.61 | 4.30 | 1.45 | 1.12 | 3.52 | 2.70 |
| 17.05 | 7.28 | 13.25 | 4.40 | 1.28 | 1.72 | 2.34 | 3.00 |
| 14.25 | 5.10 | 9.43 | 4.18 | 1.51 | 1.22 | 2.80 | 2.25 |
| 15.78 | 5.20 | ..... | 4.75 | ..... | 1.10 | 3.03 | ..... |
| 17.22 | 6.97 | ..... | 3.48 | ..... | 1.46 | 2.47 | ..... |
| 17.00 | 7.40 | ..... | 6.33 | ..... | 1.17 | 2.30 | ..... |
| 15.91 | 5.06 | ..... | 3.43 | ..... | 1.47 | 3.13 | ..... |
| 14.52 | 5.20 | ..... | 3.76 | ..... | 1.40 | 2.80 | ..... |
| 13.07 | 6.31 | ..... | 2.42 | ..... | 1.64 | 2.07 | ..... |
| 17.25 | 7.20 | ..... | ..... | ..... | ..... | 2.40 | ..... |
| 12.78 | 5.50 | ..... | ..... | ..... | ..... | 2.32 | ..... |
| ..... | 6.20 | ..... | ..... | ..... | ..... | ..... | ..... |
| ..... | 6.50 | ..... | ..... | ..... | ..... | ..... | ..... |
| ..... | 4.75 | ..... | ..... | ..... | ..... | ..... | ..... |
| ..... | 4.30 | ..... | ..... | ..... | ..... | ..... | ..... |
| ..... | 5.10 | ..... | ..... | ..... | ..... | ..... | ..... |
| ..... | 4.18 | ..... | ..... | ..... | ..... | ..... | ..... |
| Av. 15.34 | 5.64 | 11.13 | 3.95 | 1.37 | 1.43 | 2.72 | 2.87 |

### OXYGEN UPTAKE OF INTRACELLULAR CONSTITUENTS

Homogenates of embryos, prepared as indicated above, were subjected to centrifugal forces ranging from 600 to 50,000 times gravity, and fractions thus obtained were studied both cytologically and physiologically. No substrates other than those inherent in the materials were added, so that the values of oxygen uptake represent those for enzyme-substrate systems present in the embryos. The two fractionation procedures mostly employed are diagrammatically shown in Chart I.

Oxygen-uptake determinations were carried out on all precipitates and supernatants resulting from centrifugation,

CHART I




$pp_1$ = nuclei which contain enzyme $E_1$ and substrate $S_1$.
$st_1$ = supernatant which may contain enzymes $E_2$, $E_3$ and substrates $S_2$, $S_3$.
$pp_2$ = mitochondria which contain enzyme $E_2$.
$st_2$ = second supernatant which contains enzyme $E_3$ and substrates $S_2$, $S_3$.
$pp_3$ = microsomes which contain enzyme $E_3$.
$st_3$ = third supernatant which contains substrates $S_2$, $S_3$.

here (see Chart I). In general, for the morphological side of the experiments, nuclei are found in the precipitate $pp_1$ after the original homogenate has been centrifuged at 600 times gravity. Granules, both large and small, appear in the first supernatant, $St_1$, and are probably mitochondria and microsomes (?). Further centrifugation of this supernatant, $St_1$, at 1,200 times gravity gives a precipitate, $pp_2$, consisting mostly of large granules, which are undoubtedly mitochondria. The supernatant $St_2$ contains smaller granules and some small fat globules. Centrifuging supernatant $St_2$ at 1,800 times gravity gives a precipitate, $pp_3$, consisting of small granules, probably microsomes (?). The supernatant $St_3$ has in it practically no granules, but small spherical fat globules are present.

Oxygen uptake was measured and recorded for the following: intact embryos, homogenate, precipitate $pp_1$, supernatants $St_1$ and $St_2$. All other fractions showed little, if any, oxygen uptake (see Table 2 and Figs. 2 and 3). Further analyses of these fractions consuming oxygen were made and the following results noted. By heating fractions at 60° C. for periods of 10 minutes, it was possible to destroy all oxidases, and, by recombining different fractions thus treated, the presence of both enzymes and substrates could be definitely determined

Precipitate $pp_1$, containing intact nuclei, has in it both an enzyme, $E_1$, and a substrate, $S_1$, or a system of enzyme-substrate, which normally cannot be separated by washing or centrifugation. When heated at 60° C. for 10 minutes, all the enzyme is apparently inactivated, since oxygen is no longer taken up. Supernatant $St_1$ normally consumes oxygen but, when heated at 60° C. for 10 minutes, also becomes quite inactive. Precipitate $pp_2$ (mitochondria) does not normally take up oxygen but, when added to supernatants $St_1$, $St_2$, and $St_3$, shows an active oxygen uptake. When heated, $pp_2$ is quite inactivated. Supernatant $St_2$ takes up oxygen and is inactivated by exposures to 60° C. Precipitate $pp_3$, microsomes (?), does not take up oxygen but, when added to supernatant $St_3$, shows a decided uptake. Heating at 60° C. for 10 minutes completely inactivates it. By making various combinations of heated and nonheated precipitates and substrates, it can be readily shown that precipitate $pp_1$ contains an enzyme system, $E_1$, and a substrate, $S_1$, which constitute a specific enzyme-substrate system. Repeated washing of precipitate $pp_1$ failed to dissolve out substrate $S_1$, indicating its close adhesion to the enzyme withheld in the nuclei. Enzymes from precipitates $pp_2$ and $pp_3$ do not act upon substrate $S_1$. Precipitate $pp_2$ contains enzyme $E_2$, which acts upon supernatants $St_2$ and $St_3$. Precipitate $pp_3$ also acts upon supernatants $St_2$ and $St_3$. It would thus seem from these results that the enzyme-substrate system of the nuclei is more or less self-contained and does not, as such, act upon the soluble substrates of the cytoplasm. Enzymes from both mitochondria and microsomes do act upon the soluble substrates of the cytoplasm.

A slightly modified procedure from that just described is illustrated in Chart II, and results obtained by this method are similar to those for the former one.

In the foregoing procedure the formed constituents, nuclei, mitochondria, and microsomes, are completely thrown down under the influence of the high centrifugal force, while only small spherical fat globules and soluble cytoplasmic substrates remain in the supernatant (see Pl. I). Further treatment of the first precipitate according to the fractional

## TABLE 2

### OXYGEN UPTAKE BY DIFFERENT FRACTIONS AND FRACTION COMBINATIONS, CALCULATED IN PERCENTAGES, OXYGEN UPTAKE BY HOMOGENATE AS 100 PER CENT

(For Further Explanation See Text)

| Fractions | Homogenate | $pp_1$ | $St_1$ | $\Delta^* pp_1$ | $\Delta St_1$ | $pp_2$ | $St_2$ | $pp_3$ | $St_3$ | $pp_1 + pp_2$ | $pp_1 + St_2$ | $pp_1 + \Delta St_2$ | $\Delta pp_1 + St_2$ | $\Delta pp_1 + pp_2$ | $pp_1 + St_1$ | $pp_2 + St_2$ | $pp_3 + St_3$ | $pp_2 + St_1$ | $pp_2 + St_2$ | $pp_3 + St_1$ | $pp_3 + St_2$ | $pp_3 + pp_1$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 100 | 42 | 50 | 0 | 0 | 0 | 4 | 6 | 0 | 62 | 44 | 70 | 8 | 0 | 90 | 35 | 19 | 61 | 34 | 47 | 16 | 60 |
| | 100 | 56 | 36 | 12 | 3 | 10 | 5 | 5 | 0 | 55 | 62 | 65 | 5 | 10 | 80 | 46 | 12 | 80 | 48 | 50 | 15 | 62 |
| | 100 | 43 | ... | 9 | 5 | 22 | 2 | 0 | 3 | 40 | 44 | 45 | ... | 7 | 86 | 48 | ... | ... | 26 | ... | 35 | 60 |
| | 100 | 52 | ... | ... | ... | 15 | 10 | 0 | 0 | 56 | 45 | 60 | ... | 10 | ... | ... | ... | ... | ... | ... | ... | ... |
| | 100 | 52 | ... | ... | ... | 4 | 13 | 0 | 0 | ... | 51 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| | 100 | 57 | ... | ... | ... | 10 | 2 | 0 | 0 | ... | 46 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| | 100 | 60 | ... | ... | ... | 9 | ... | ... | ... | ... | 70 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| | 100 | 60 | ... | ... | ... | 0 | ... | ... | ... | ... | 76 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| | 100 | 66 | ... | ... | ... | 16 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| | 100 | 78 | ... | ... | ... | 17 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| | 100 | 68 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| Sum... | 1,100 | 634 | 86 | 21 | 8 | 103 | 36 | 11 | 3 | 213 | 438 | 240 | 13 | 27 | 256 | 129 | 31 | 141 | 108 | 97 | 66 | 182 |
| Av..... | 100 | 57 | 43 | 7 | 3 | 10 | 6 | 2 | 1 | 52 | 55 | 60 | 7 | 9 | 82 | 43 | 16 | 71 | 36 | 48 | 22 | 61 |

* Δ = fraction heated at 60° C. for periods of 10 minutes.

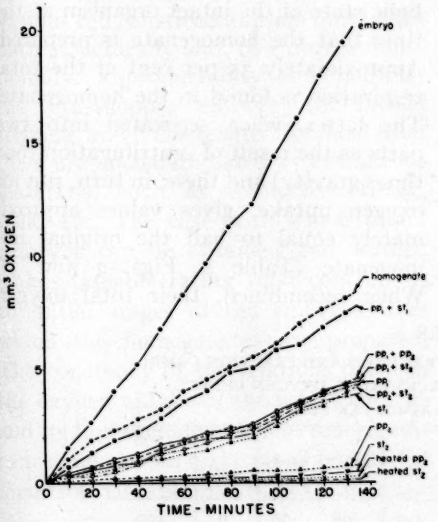



FIG. 2.—Shows oxygen uptake for intact post-diapause embryos, their homogenate and intracellular constituents. For explanations of abbreviations see Chart I.

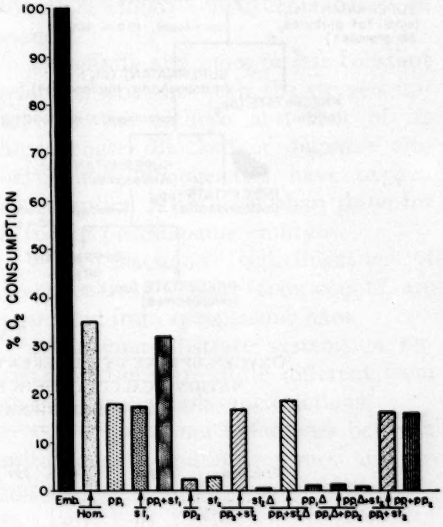
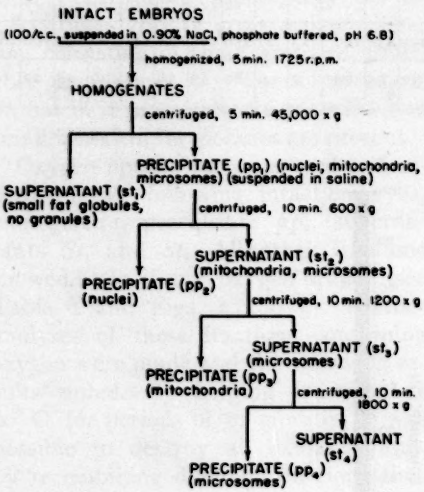



FIG. 3.—Shows oxygen uptake in terms of intact embryo as 100 per cent of homogenate and intracellular constituents. For further description see text and Chart I.

centrifugation previously employed permits an almost complete separation of the formed constituents. The nuclear fraction so obtained, even after thorough washing, still consumes oxygen, indicating the presence of an enzyme-substrate system within the nuclei. Mitochondria and microsome fractions, when added to the original supernatant, consume oxygen, as previously pointed out. Functionally, no results differing from those obtained by the first procedure have been noted (see Table 3).

CHART II


INTACT EMBRYOS
(100/c.c., suspended in 0.90% NaCl, phosphate buffered, pH 6.8)
homogenized, 5 min. 1725 r.p.m.
HOMOGENATES
centrifuged, 5 min. 45,000 x g
PRECIPITATE (pp1) (nuclei, mitochondria, microsomes) (suspended in saline)
SUPERNATANT (st1) (small fat globules, no granules)
centrifuged, 10 min. 600 x g
SUPERNATANT (st2) (mitochondria, microsomes)
PRECIPITATE (pp2) (nuclei)
centrifuged, 10 min. 1200 x g
SUPERNATANT (st3) (microsomes)
PRECIPITATE (pp3) (mitochondria)
centrifuged, 10 min. 1800 x g
SUPERNATANT (st4)
PRECIPITATE (pp4) (microsomes)

ENZYME-SUBSTRATE SYSTEMS; THEIR QUANTITATIVE RELATIONS

Among the criticisms of the fractional centrifugation techniques, that of the inability to make the reactions of the sum of the parts equal to that of the whole seems to be of major significance (Schneider, 1949). In the present investigations with a more or less simple invertebrate material, rigidly maintained and standardized, it has been possible to evaluate the reactions of the constituent parts and to compare them with the intact organism and its homogenates. The results of such experiments are given in Tables 1, 2, and 3 and in Figures 1, 2, and 3. If one considers the oxygen uptake of the intact embryo as 100 per cent, its homogenate bears a more or less constant relation to it and seems to depend for its absolute value upon the metabolic state of the intact organism at the time that the homogenate is prepared. Approximately 35 per cent of the total respiration is found in the homogenate. The latter, when separated into two parts as the result of centrifugation (600 times gravity) and these, in turn, run for oxygen uptake, gives values approximately equal to half the original homogenate (Table 2; Figs. 2 and 3). When recombined, their total oxygen

TABLE 3

OXYGEN UPTAKE BY DIFFERENT FRACTIONS AND FRACTION COMBINATIONS, CALCULATED IN PERCENTAGES, OXYGEN UPTAKE BY HOMOGENATE AS 100 PER CENT

| Fractions | $pp_1$ | $Sl_1$ | $pp_2$ | $Sl_2$ | $pp_2+Sl_1$ | $pp_2+\Delta Sl_1$* | $\Delta Sl_1$ | Homogenate |
|---|---|---|---|---|---|---|---|---|
| | 68 | 4.9 | 56 | 4.7 | 98 | 79 | 0 | 100 |
| | 68 | 6.7 | 56 | 8.4 | 93 | 88 | 0 | 100 |
| | 69 | 4.7 | 62 | 0 | 85 | 100 | ..... | 100 |
| | 69 | 4.0 | 61 | 11 | 94 | ..... | ..... | 100 |
| | 67 | ..... | 63 | ..... | 118 | ..... | ..... | 100 |
| Sum..... | 341 | 20.3 | 353 | 24.1 | 488 | 267 | 0 | 500 |
| Av..... | 68 | 5 | 59 | 6 | 98 | 89 | 0 | 100 |

* $\Delta$ = fraction heated at 60° C. for 10 minutes.

uptake is practically equal to that of the homogenate from which they were formed (Fig. 3). As pointed out above, the nuclei-containing fraction apparently has a substrate closely associated with it, and this, for any given homogenate, is more or less constant, as judged by the oxygen uptake of the fraction. The mitochondria, when washed, do not consume oxygen unless added to a supernatant containing soluble cytoplasmic substrate. Microsomes react functionally similarly to mitochondria, and one may assume from this material that they represent smaller particles resembling mitochondria in their reactions.

## DISCUSSION

Comparisons between the present data and those for vertebrate tissues show marked similarities, in that the various cell constituents seem quite alike both structurally and, to a degree, functionally. Reproducibility of results for the grasshopper embryo are indeed striking, and homogenates obtained from them are uncontaminated by extraneous materials, such as connective tissue, yolk, etc. The ease with which intact embryos can be obtained and studied makes possible rather rigid controls as to initial stages of the embryos from which the homogenates are prepared. The constancy of the relations between the oxygen uptake of the intact embryo and its homogenate seems of real significance and should make possible detailed quantitative experiments as to the basic differences involved in the various physiological states of the organisms.

It seems reasonable to conclude that, for the homogenates of the grasshopper embryo, at least two or three different enzyme-substrate systems are present. Enzymes from the nuclei apparently do not act upon soluble substrates of the cytoplasmic fractions. Functionally, no marked or definite differences between the enzymes of the mitochondria and those of the microsome have been found. Further studies may throw light upon the basic physiochemical differences between the active and blocked states of these organisms.

## SUMMARY

1. A study of the oxygen uptake of the intact embryo of the grasshopper, *M. differentialis*, its homogenates, and the intracellular constituents has been made.

2. Definite and more or less constant relations exist between the oxygen uptake of the embryo and that of its homogenate. Blocked or diapause embryos and homogenates have oxygen consumption rates lower than those for active or postdiapause embryos.

3. By fractional centrifugation of homogenates, nuclear components are separated from cytoplasmic ones.

4. Enzyme-substrate systems of nuclear fractions are quite different from those of the cytoplasmic fractions.

5. No functional differences between mitochondria and microsomes are apparent.

## LITERATURE CITED

BENSLEY, R. R., and HOERR, N. L. 1934*a*. Studies on cell structure by the freezing-drying method. V. The chemical basis of the organization of the cell. Anat. Rec., 60:251–66.

———. 1934*b*. Studies on cell structure by the freezing-drying method. VI. The preparation and properties of mitochondria. *Ibid.*, pp. 449–55.

BODINE, J. H. 1934. The effect of cyanide on the oxygen consumption of normal and blocked embryonic cells (Orthoptera). Jour. Cell. and Comp. Physiol., 4:397–404.

———. 1950*a*. To what extent is oxygen uptake of the intact embryo related to that of its homogenate? Science 112:110–11.

BODINE, J. H. 1950*b*. The combined action of 2, 4-dinitrophenol and ethyl carbamate (urethane) on the oxygen uptake of embryonic cells. Physiol. Zoöl., 23:63–67.

POTTER, V. R., and ELVEHJEM, C. A. 1936. A modified method for the study of tissue oxidation. Jour. Biol. Chem., 114:495–504.

SCHNEIDER, W. C. 1946. Intracellular distribution of enzymes. I. The distribution of succinic dehydrogenase, cytochrome oxidase, adenosinetriphosphatase, and phosphorus compounds in normal rat tissues. Jour. Biol. Chem., 165:585–93.

———. 1949. The distribution of enzymes within the cell. In: LARDY, H. A., Respiratory enzymes. Minneapolis, Minn.: Burgess Pub. Co. (Contains more or less complete bibliography.)

SCHNEIDER, W. C.; CLAUDE, A.; and HOGEBOOM, G. H. 1948. The distribution of cytochrome C and succinoxidase activity in rat liver fractions. Jour. Biol. Chem., 172:451–58.

SLIFER, E. H. 1931. Insect development. II. Mitotic activity in the grasshopper embryo. Jour. Morphol., 51:613–18.

PLATE I

FIG. *A*.—Nuclear fraction, $pp_1$. ×450.
FIG. *B*.—Mitochondrial fraction, $pp_2$. ×450.
FIG. *C*.—Microsomal fraction, $pp_3$. ×450.
FIG. *D*.—Supernatant, containing fat globules. ×450.

# A CYTOLOGICAL AND CYTOCHEMICAL STUDY OF THE TESTICULAR CYST-CELLS IN THE JAPANESE BEETLE

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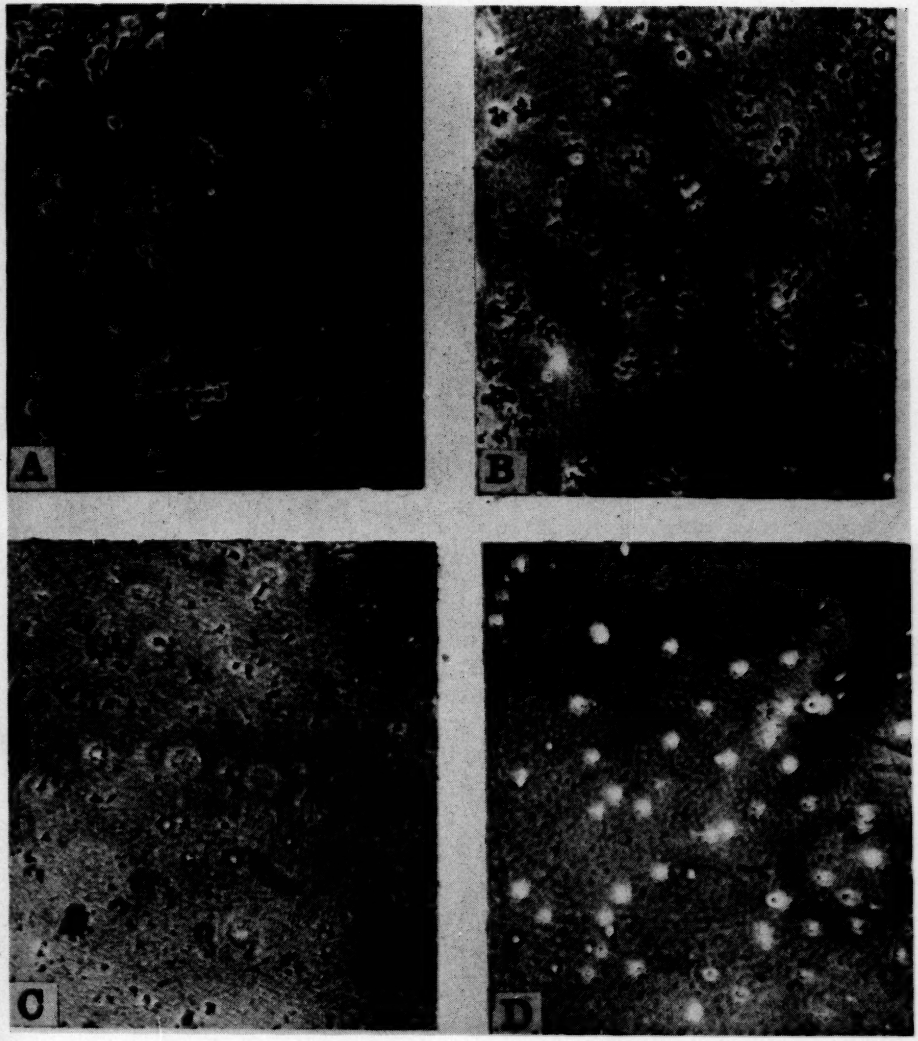
THE behavior of testicular cyst-cells and their relationship to the developing spermatozoa in the arthropod testis presents an interesting problem. In the Japanese beetle these cells form envelopes about groups of spermatocytes and continue to inclose the maturing gametes until the release of the spermatozoa in the vas efferens. The cyst-cells then reorganize and accompany the spermatozoa through the effluent passages and into the bursa copulatrix of the female, where they disintegrate.

This paper reports the results of studies on the cytology and cytochemistry of the cyst-cells during their period of intimate relationship with the developing spermatozoa and during their subsequent degenerative changes.

The general occurrence of nongerminal supporting cells in the testes of animals has long been known. Gilson (1884) described and illustrated cyst-cell envelopes surrounding packets of spermatozoa in many arthropods and showed clearly the attachment of gametes to these cells in the later stages of their development. In a discussion of the occurrence and significance of cyst-cells in animals, Grobben (1899) criticized Gilson's application of the term "conjugation" to this attachment and proposed that the supporting cells, variously known as "foot-cells," "basal cells," and "female elements," be termed *Samennährzellen*. The nutritive function of the cyst-cells, implied in Grobben's terminology, has never been established, and the physiological significance of the relationship between cyst-cell and gametes remains in doubt.

Cyst-cells in Scarabaeoid beetles have been observed by Gilson (1884) in *Geotrupes* and others, by Prowazek (1902) in *Oryctes*, and by Rittershaus (1927) in *Phyllopertha* and *Anomala*, members of the same family (Rutelidae) as *Popillia japonica*, the Japanese beetle.

PLATE I



## MATERIAL AND METHODS

Entire reproductive tracts were dissected from adult male Japanese beetles; individual testes were removed from prepupae and from pupae in various stages of development. These were fixed in a variety of fluids, depending on the technique to be followed, and were either imbedded in paraffin and sectioned serially at 5 $\mu$ or imbedded in gelatin and sectioned on the freezing microtome.

For morphological relationships and some cytological detail, material was fixed in Carnoy-Lebrun or in strong Flemming and stained with iron-alum haematoxylin, or in Zenker-acetic followed by methylene blue–eosin. Baker and Thomas' osmic-bichromate fixative (Lee, 1937, p. 35) and iron-alum haematoxylin were used to demonstrate mitochondria. Nuclear and cytoplasmic basophilia was studied in material fixed in Zenker-acetic and stained with eosin–methylene blue; this technique was controlled by incubation of adjacent sections with ribonuclease.[1] Feulgen preparations were also used to demonstrate desoxyribonucleoproteins. For the study of lipid elements, Baker's sudan-black technique (1944) and Cain's method involving postchromation followed by sudan black (1947) were employed. Phospholipids were localized by Baker's acid hematein technique (1946). Glycogen and other polysaccharides were studied by the periodic acid–leucofuchsin routine of Hotchkiss (1948); control sections were treated with saliva to remove glycogen. Special methods for the demonstration of the Golgi element included Worley's osmication technique (1946) and the DaFano silver method, in addition to the sudan-black techniques. Acid and alkaline phosphatase methods were also employed (Gomori, 1941; Wolf *et al.*, 1943) with a variety of substrates. For the detection of metachromatic substances, material was fixed in basic lead acetate–formaldehyde and stained with a 0.1 per cent solution of toluidine blue in 1 per cent alcohol.

[1] Supplied by Dr. William Montagna from material prepared in this laboratory. This preparation was freed of proteolytic activity by prolonged heating at 60° C.

## OBSERVATIONS

### A. MORPHOLOGICAL AND GENERAL

Each of the testes of the beetle is a somewhat flattened, globose structure lying about the terminal funnel of an individual vas efferens (Anderson, 1949; cf. Bordas, 1900, and Rittershaus, 1927, for other Scarabaeoids). The vasa efferentia from the six testes on each side of the body unite to form a vas deferens. The two vasa deferentia proceed posteriad and mediad to join with the paired accessory reproductive glands and form the ejaculatory duct.

Each testis is divided into a number of radial segments (follicles), separated from one another by thin connective-tissue partitions. An apical proliferative region lies above the central mouth of the vas efferens funnel. Groups of spermatocytes produced in this gonial region move outward and downward through the follicles, passing along the outer wall of the follicle as development proceeds. Groups of spermatids usually lie at the inner and lower ends of the follicles, below and lateral to the lip of the funnel. Metamorphosis of the spermatozoa occurs in this region, and the packets then move upward and into the roof of the funnel cavity below the apical region. Here the gametes are released from their envelopes and proceed independently into the vas efferens, along with the rounded-up cyst-cells.

Throughout the period of their intimate relationship with the developing

gametes, the cyst-cells are stretched into a very thin layer over the inclosed cells. As elongation of the sperm nuclei progresses, with the formation of the definitive acrosome of the sperm head, orientation of the metamorphosing spermatids with their heads at one end of the bundle is completed. Elongation of the tails leads, finally, to the elimination from each spermatid of a small mass of residual cytoplasm containing the Golgi remnant (ex-acroblast). These masses of cytoplasm are apparently taken up by the cyst-cells.

Slow, unorganized undulations of the individual spermatozoa, including their nuclear portions, now begin and bring about movement of the spermatozoa into a perfect alignment of heads and acrosomes, side by side and pressed closely together. The assumption of this regimented appearance marks the entry of the acrosomes into the cytoplasm of the nearest cyst-cell. At this time the movements of the individual spermatozoa cease. The terminal acrosomal granules of all the spermatozoa lie close together in a small area of cyst-cell cytoplasm (Fig. 1). Apparently very soon after the establishment of this relationship, coordinated undulations of the sperm tails begin and thereafter increase in rate. The sperm bundle is still inclosed by the cyst-cell envelope, but this envelope is so thin and follows so closely the contours of the sperm mass within that these undulations succeed in propelling the bundle through the crowded follicle and into the roof of the funnel cavity.

The subsequent release of the spermatozoa from the envelope is evidently sudden, and all spermatozoa apparently withdraw simultaneously from the cyst-cell. The abandoned envelope gradually reverts to the two cells of which it was composed. The further history of these cells consists of progressive degenerative changes, in which the cytoplasm becomes increasingly vacuolated and the nuclei become shrunken and pyknotic.

### B. THE CYST-CELLS DURING SPERM FORMATION

The cyst-cells show a marked degree of cytoplasmic basophilia throughout the period during which they inclose developing spermatozoa. This basophilia is more or less uniform throughout the cytoplasm (Fig. 2). Chromatin masses in the cyst-cell nuclei are strongly basophilic and give a positive Feulgen reaction. All the cytoplasmic basophilia and some of the nuclear basophilia are removed by treatment with ribonuclease (Fig. 3).

The cytoplasm of the cyst-cells is rich in glycogen during this period, as demonstrated by the periodic acid–leucofuchsin technique (Fig. 4). Most of the material responsible for the recoloration of Schiff's reagent is removed by salivary digestion, leaving only a small amount of reactivity, apparently due to the presence of other polysaccharides in the cytoplasm. The residual material shows no metachromasia when stained with toluidine blue. The glycogen appears to be somewhat more concentrated in the region immediately surrounding the point of attachment of the spermatozoa during the late stages of the development.

Owing to the thinness of the cytoplasmic layer comprising the cyst-cell envelope, lipid elements in the cyst-cell are difficult to demonstrate during the developmental stages. However, late in the period of sperm development, large globules of lipoid material may be observed after treatment with osmium or with sudan black. These elements lie near the posterior ends of the bundles and probably represent degenerating lipid bodies brought into the cyst-cell with the residual cytoplasm from the spermatids.

Alkaline phosphatase activity was observed in sections incubated with hexose diphosphate at pH 9.4. This activity was restricted to the terminal acrosomal granules of the imbedded sperm tips and to the region of the cyst-cell cytoplasm immediately surrounding them and appeared to spread diffusely over the surfaces of the sperm heads (Fig. 5). No phosphatase activity in these areas was found following incubation with adenylic acid, nucleic acid, or beta-glycerophosphate at pH 9.4. Acid phosphatase could not be demonstrated in the cyst-cells, with the use, as substrates, of beta-glycerophosphate and a mixture of glycerophosphate and adenylic acid.

### C. THE CYST-CELLS FOLLOWING RELEASE OF SPERMATOZOA

The rounded-up cyst-cells in the vas efferens funnel shortly after the release of the spermatozoa are uniformly ovoid, their nuclei are smooth and regular in outline, and they appear normal in every way. At this stage the cytoplasmic basophilia remains moderately strong (Fig. 2), and the basophilia and Feulgen reactivity of the nuclear contents appear to have undergone no change.

The glycogen content of the cyst-cells is remarkable. During their sojourn in the funnel and in the short vas efferens, the cyst-cells show masses of Schiff-positive material which is completely removed by salivary digestion (Fig. 7).

Both sudan-black and osmium methods demonstrate lipid bodies in the cells at this stage. Small to moderate-sized clear vacuoles, each surrounded by an irregular sudanophilic and osmiophilic rim, lie in the cytoplasm near the nucleus (Figs. 6, 8, 9). Many minute granular bodies dispersed in the cytoplasm are also colored by both techniques. In addition, many cells show a large vacuolar body containing irregularly distributed peripheral, blackened clumps (Fig. 6). These appear identical with the bodies observed earlier in the posterior ends of cyst-cell envelopes. The use of sudan black following postchromation of the material shows essentially the same bodies as revealed without postchromation (Fig. 10).

With the acid hematein technique, the cyst-cells show small to moderate-sized stained bodies in the cytoplasm (Fig. 11). In general, these are similar to those shown by sudan black but are less intensely colored. These structures are absent from cyst-cells extracted with hot pyridine.

Following the release of the spermatozoa, there is no alkaline phosphatase activity in the cyst-cells.

As the cyst-cells proceed down the vasa deferentia and into the seminal vesicles at their lower ends, conspicuous signs of degeneration develop. The cytoplasm progressively loses its basophilia and becomes increasingly vacuolated (cf. cells in Figs. 2 and 12). In addition, the basophilia remaining in the nucleus of each cell following treatment with ribonuclease becomes less and less intense. Feulgen preparations of cells at this level also reveal that Feulgen-positive material in the nucleus steadily decreases.

The large lipid-containing structures noted in cells in the funnel and vas efferens disappear by the time the cyst-cells reach the seminal vesicle. Certain irregularly shaped fragments of the smaller sudanophilic and osmiophilic bodies remain, however, in the cytoplasm between the large vacuoles. At this stage they do not consistently appear to contain vacuoles, although occasional postchromed sudan-black preparations show, near the nucleus, one or more vacuoles with blackened rims.

As the cyst-cells pass down the vas deferens, their cytoplasm appears to be-

come increasingly packed with glycogen (Fig. 13). The glycogen masses seem to coalesce and to occupy the large vacuoles shown in cells at this level in all types of preparations. A cyst-cell lying in the lowest portion of the vas deferens, at the point of junction of the accessory reproductive glands, is practically a mass of glycogen inclosed by a cell membrane and surrounding a nucleus which has lost almost all its basophilia.

## DISCUSSION

These observations may partly explain the functions of the cyst-cells in the insect testis and may indirectly explain the possible role of the acrosome in the later stages of the development of the spermatozoön.

Following the suggestion of Grobben (1899) that the cyst-cells functioned in the nutrition of the developing spermatozoa, Montgomery (1911) ventured the opinion that the major function of the acrosome in the insect sperm might involve the transfer of nutrients from cyst-cell to spermatozoön rather than penetration of the egg. Bowen (1924), while admitting the possibility that this might be *one* function of the acrosome, inclined toward the belief that its *chief* function was to furnish certain enzymes which assist the penetration of the egg by the spermatozoön. Bowen had previously rejected the "cutting-tool" interpretation of the nature of the acrosome.

It is useless to speculate at this time as to the relative importance of the various functions of the acrosome. However, from observations on the cyst-cells and spermatozoa in the present studies, some significant points of evidence as to their several functions may be pointed out.

The continued presence of cytoplasmic basophilia in the cyst-cells during the period in which they form envelopes about sperm masses may be taken to indicate that significant synthetic activities are proceeding in the cytoplasm. There is abundant evidence that this type of basophilia, depending upon the presence in the cytoplasm of deposits of ribonucleoproteins, is associated with active synthetic processes within the cell. The continued and progressive accumulation of glycogen within the cyst-cells furnishes clear evidence that these cells function in the storage of nutritional reserves. This fact, plus the demonstration of phosphatase activity in the terminal granules of the imbedded sperm tips, suggests very strongly that transfer of some of this nutrient material from cyst-cell to spermatozoa, mediated through the acrosome, occurs during the attached phase of development. It is perhaps significant that the establishment of the intimate relationship between cyst-cell and spermatozoa coincides with the elimination from the late spermatids of all residual cytoplasm, leaving the spermatozoa without demonstrable intrinsic stores of energy-rich substances.

The analogy between the cyst-cells of the insect testis and the Sertoli cells of the vertebrate testis is apparent. Both function as points of attachment of late spermatids during their metamorphosis into finished spermatozoa. Recent work by Wislocki (1949) on the testis of deer emphasizes other points of similarity. It has been demonstrated in this material that the Sertoli cells contain glycogen. In addition, alkaline phosphatase activity was found in the cytoplasmic membrane surrounding the sperm head during its attached phase and in the cytoplasm of the Sertoli cells between germinal cells, especially in regions where spermatids and spermatozoa are attached.

It is also significant that the developing spermatozoa of the Japanese beetle

never show co-ordinated beating, as a group, until after their acrosomes have become attached to the cyst-cell. The undulations of the individual spermatozoa before attachment are always random and unregulated, but the beating of the tails after penetration has occurred is always perfectly co-ordinated (Fig. 14). This may, of course, reflect a purely chronological relationship between time of attachment and time of commencement of co-ordinated beating. It seems more likely, however, that one of the functions of the side-by-side alignment of the acrosomal granules within the cytoplasm of the cyst-cell is to bring about the co-ordination of beat necessary for the most efficient propulsion of the sperm bundle as a unit.

The vacuolar structures noted in the cyst-cells just after the withdrawal of the spermatozoa resemble very strongly the Golgi element, as shown by sudan-black methods in other tissues of the Japanese beetle. The smaller granular, blackened bodies may be interpreted as mitochondrial elements, as these are demonstrated equally well by chrome-osmic-haematoxylin and by the use of sudan black. The nature of the large blackened vacuolar elements is problematic. However, from the time and place of their appearance and from the fact that they shortly disappear, it may be

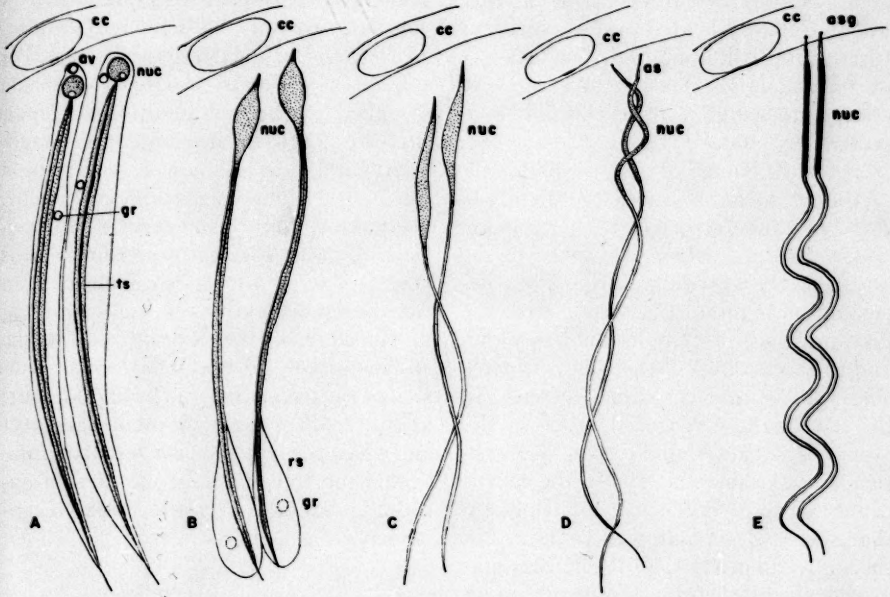



FIG. 14.—Diagrammatic representation of stages in development of spermatozoa and of the relationship between spermatozoa and cyst-cells. Only two spermatozoa are shown in each cyst, and the tails are represented as proportionally much shorter than they actually are. Abbreviations: *cc*, cyst-cell; *nuc*, nucleus; *av*, acrosomic vesicle; *gr*, Golgi remnant; *ts*, tail sheath; *rs*, residual cytoplasmic sheath; *as*, acrosome; *asg*, acrosomic granule. *A*, late spermatids; *B–C*, stages in elongation of head and development of acrosome, loss of residual cytoplasm and its contents, continued growth of tail; *D*, maturing spermatozoa (note irregular undulations of nuclear and tail portions and diffuse appearance of chromatin in nuclei); *E*, mature, attached spermatozoa; acrosomic granules imbedded in cytoplasm of cyst-cell; nuclear portions quiescent and regularly aligned; chromatin dense. Note co-ordination in beating tails.

assumed that these represent degenerative stages in the disposal of the coalesced Golgi remnants of the late spermatids.

The tendency of cytoplasmic bodies resembling parts of the Golgi element and possibly mitochondria to stain with acid hematein and to be extracted with hot pyridine indicates that these structures contain phospholipid. The presence of phospholipid in such structures in cells of other organisms has been demonstrated by Baker (1946), Cain (1947, 1948), and Thomas (1947) and in cells of the accessory reproductive glands of *Popillia* (Anderson, 1950).

The loss of Feulgen reactivity in the nuclei of the cyst-cells during their degeneration is interesting along with the disappearance of cytoplasmic basophilia and the breakdown of the Golgi element. The concomitant apparent increase in the size of the glycogen deposits in the cyst-cells suggests that the major portion of the usable material in the cells is converted to glycogen; it seems unlikely that such a degenerate cell could retain the ability to produce glycogen from materials absorbed from the surrounding medium.

The cyst-cel's disintegrate in the lower portions of the female genital tract. The stores of glycogen thus released presumably serve in the maintenance of vitality of the masses of spermatozoa which are passed into the seminal receptacle for storage. It has been shown (Anderson, 1950) that the secretion of the accessory reproductive glands of the male, which, together with the spermatozoa and cyst-cells, makes up the seminal fluid of the beetle, contains no glycogen but large amounts of mucopolysaccharides. The contributions of glycogen from the disintegrating cyst-cells may then be an important source of nutrition for stored spermatozoa.

The probable functions of the cyst-cells may be summarized as follows: (*a*) during developmental stages of the spermatocytes and spermatids, to concentrate large stores of glycogen, which are presumably used in the nutrition of the developing cells within; (*b*) to bind together groups of cells in similar stages of maturation, so that large numbers of spermatozoa may be delivered simultaneously to the vas efferens; (*c*) to gather up and dispose of the residual cytoplasm sloughed off by metamorphosing spermatozoa; (*d*) to furnish a point of attachment for the acrosomes of spermatozoa during their final stages of development; presumably to furnish energy-rich materials for the spermatozoa during this stage; possibly to furnish a mechanism for the co-ordination of the swimming movements of the individuals in the sperm packet, so that effective propulsion of the packet may be brought about; and (*e*) to disintegrate in the female tract and thus possibly furnish nutritive materials for the maintenance of spermatozoa in storage in the seminal receptacle.

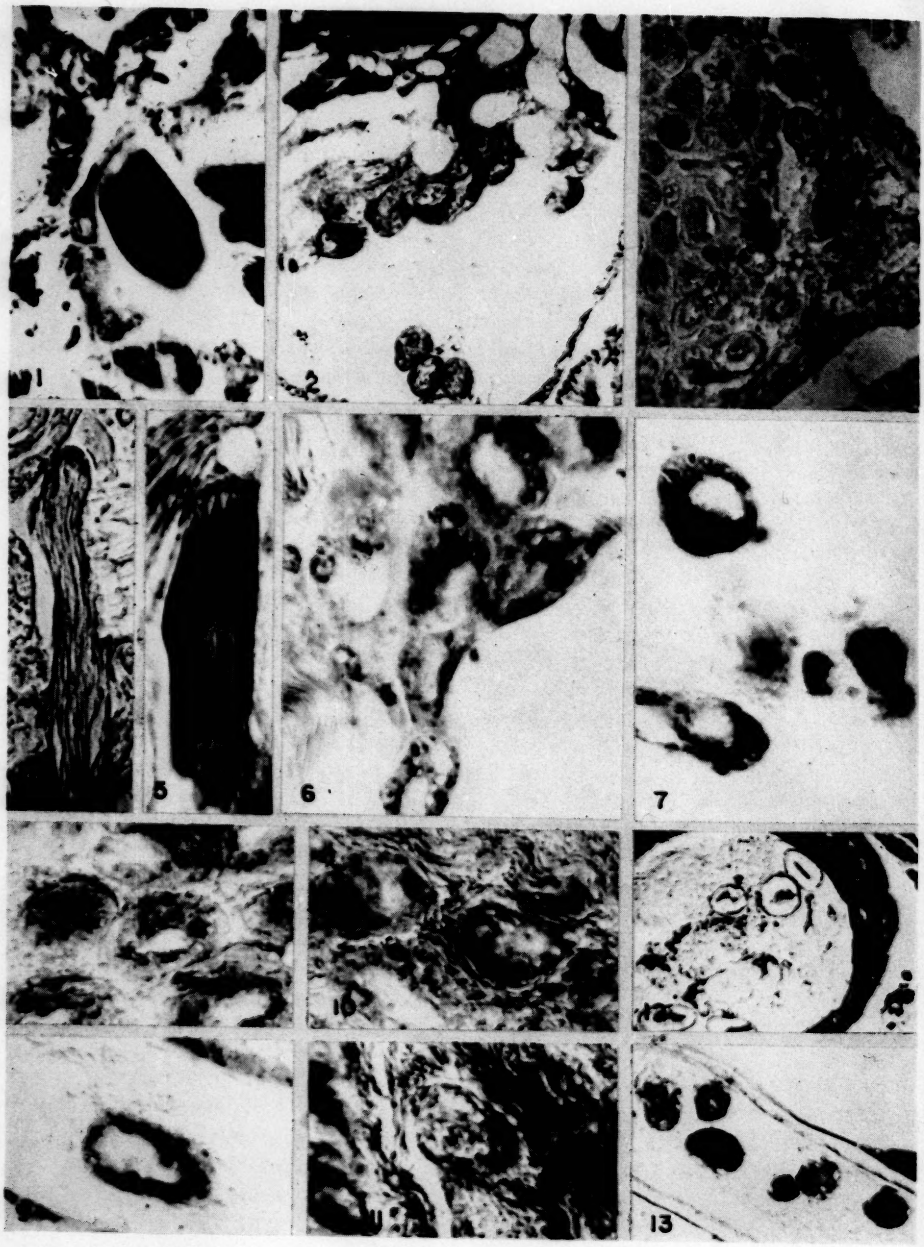
## SUMMARY

During spermatogenesis in *Popillia*, testicular cyst-cells form envelopes about groups of spermatocytes and continue to surround developing gametes until they are mature. As spermatozoa finish their development, they attach by their acrosomes to the cytoplasm of a cyst-cell.

Cyst-cells contain cytoplasmic deposits of ribonucleoproteins, large amounts of glycogen, and lipid bodies interpreted as the Golgi element and mitochondria. The lipid bodies, at least in part, react positively in tests for phospholipids.

Release of spermatozoa from their cyst-cell envelopes in the vas efferens funnel initiates degenerative changes in the abandoned cyst-cells. Cytoplasmic basophilia disappears, nuclear basophilia

PLATE I



is lost as the nucleus becomes pyknotic, and the lipid elements disintegrate. Cytoplasmic stores of glycogen increase, however, and persist until the disintegration of the cyst-cells in the female tract following copulation.

Terminal acrosomal granules of the sperm tips imbedded in glycogen-rich cyst-cell cytoplasm show alkaline phosphatase activity. This is interpreted as indicating transfer of nutrients from cyst-cell to spermatozoa, which at this time have shed their residual cytoplasm and lack demonstrable stores of glycogen.

The co-ordinated beating of the sperm tails of one bundle commences with the establishment of the attachment to the cyst-cell. This may indicate that another function of the attachment is to mediate integrating influences between individual spermatozoa and bring about co-ordinated swimming movements which propel the bundle through the follicle to the vas efferens funnel.

Glycogen stores released by disintegration of the cyst-cells in the female tract may supply nutriment for the maintenance of spermatozoa stored in the seminal receptacle.

## LITERATURE CITED


ANDERSON, J. M. 1949. Observations on spermatogenesis in the Japanese beetle, *Popillia japonica* Newman. Anat. Rec., **103**:110–11 (abstr.).

———. 1950. A cytological and cytochemical study of the male accessory reproductive glands of the Japanese beetle, *Popillia japonica* Newman. Biol. Bull., **99**: (in press).

BAKER, J. R. 1944. The structure and chemical composition of the Golgi element. Quart. Jour. Micr. Sci., **85**:1–71.

———. 1946. The histochemical recognition of lipine. *Ibid.*, **87**:441–70.

BORDAS, L. 1900. Recherches sur les organes reproducteurs mâles des Coléoptères (anatomie comparée, histologie, matière fécondante). Ann. sci. nat., sér. 8, **11**:283–448.

BOWEN, R. H. 1924. On the acrosome of the animal sperm. Anat. Rec., **28**:1–13.

CAIN, A. J. 1947. Demonstration of lipine in the Golgi apparatus in gut cells of *Glossiphonia*. Quart. Jour. Micr. Sci., **88**:151–57.

———. 1948. The accumulation of carotenoids in the Golgi apparatus of neurones of *Helix*, *Planorbis*, and *Limnaea*. *Ibid.*, **89**:421–28.

GILSON, G. 1884. Étude comparée de la spermatogénèse chez les Arthropodes. Cellule, **1**:1–188.

GOMORI, G. 1941. The distribution of phosphatase in normal organs and tissues. Jour. Cell. and Comp. Physiol., **17**:77–83.

GROBBEN, K. 1899. Über die Anordnung der Samenkörper zu Bündeln im Hoden vieler Thiere, sowie deren Ursache. Zool. Anz., **22**:104–8.

HOTCHKISS, R. D. 1948. A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. Arch. Biochem., **16**:131–41.

LEE, A. B. 1937. The microtomist's vade-mecum. 10th ed. Philadelphia: Blakiston Co.

MONTGOMERY, T. H., JR. 1911. The spermatogenesis of an Hemipteron, *Euschistus*. Jour. Morphol., **22**:731–818.

PROWAZEK, S. 1902. Spermatologische Studien. II. Spermatogenese des Nashornkäfers (*Oryctes nasicornis* L.). Arb. Zool. Inst. Wien, **13**:223–36.

RITTERSHAUS, K. 1927. Studien zur Morphologie und Biologie von *Phyllopertha horticola* L. und *Anomala aenea* Geer (Coleopt.). Zeitschr. f. Morphol. u. Oekol. Tiere, **8**:271–408.

THOMAS, O. L. 1947. The cytology of the neurones of *Helix aspersa*. Quart. Jour. Micr. Sci., **88**: 445–62.

WISLOCKI, G. B. 1949. Seasonal changes in the testes, epididimides, and seminal vesicles of deer investigated by histochemical methods. Endocrinology, **44**:167–89.

WOLF, A. E.; KABAT, A.; and NEWMAN, W. 1943. Histochemical studies on tissue enzymes. III. A study of the distribution of acid phosphatases with special reference to the nervous system. Am. Jour. Path., **19**:423–39.

WORLEY, L. G. 1946. The Golgi apparatus—an interpretation of its structure and significance. Ann. New York Acad. Sci., **47**:1–56.


## PLATE I

Figures 6, 8, 9, 10, and 11 are of frozen sections; all others are of paraffin-imbedded material, sectioned at 5 $\mu$. Magnifications given are of the figures as they appear here.

FIG. 1.—Portion of cyst-cell, showing nucleus and row of acrosomal granules of imbedded sperm tips. Carnoy-Lebrun, iron-alum haematoxylin. 1,155×.

FIG. 2.—Vas efferens funnel cavity and portion of its roof. Zenker-acetic, eosin-Y, and methylene blue. Note cytoplasmic and nuclear basophilia of cyst-cells surrounding sperm

bundles (*upper right;* light areas are groups of sperm tails) and after release of spermatozoa (*center, and free in funnel*). The strongly basophilic cells at upper left are gonia in the apical proliferative region. 436×.

FIG. 3.—Similar to section shown in Fig. 2; preparation as in Fig. 2, but exposed to ribonuclease. Note loss of cytoplasmic basophilia and reduction in intensity of nuclear basophilia in cyst-cells. 436×.

FIG. 4.—Glycogen in cyst-cell envelope. Longitudinal section of maturing sperm bundle, periodic acid–Schiff. Note concentration of glycogen-rich cyst-cell cytoplasm at head end of bundle (*upper*). 436×.

FIG. 5.—Alkaline phosphatase in acrosomal granules and neighboring areas of cyst-cell cytoplasm. Gomori method, hexose diphosphate at pH 9.4. Nuclear portions of sperm heads counterstained (cf. Fig. 1). 1,155×.

FIG. 6.—Sudanophilic bodies in cyst-cells, roof of funnel cavity. Calcium formaldehyde, sudan black. Note scattered small vacuoles, large blackened spheres. 1,155×.

FIG. 7.—Glycogen in cyst-cells in vas efferens funnel. Periodic acid–Schiff. Black spots and small clear spheres are refractile bodies of unknown significance. 1,155×.

FIG. 8.—Osmiophilic bodies in cyst-cells, funnel cavity. Worley preparation. Compare with Fig. 6. 1,155×.

FIG. 9.—Sudanophilia in cyst-cell, upper part of vas efferens. Calcium formaldehyde, sudan black. Note clumped vacuolar structures and absence of large spheres shown in Fig. 6. Cf. Figs. 8 and 10. 1,155×.

FIG. 10.—Sudanophilia after postchromation, cyst-cell in vas efferens. Calcium formaldehyde, dichromate calcium, sudan black. Note vacuolar structures and scattered blackened granules. 1,155×.

FIG. 11.—Phosphilipids in cyst-cell, upper end of vas deferens. Acid hematein technique. Note localization of stained material in rims of vacuoles and in granules at one end of cell (cf. preceding figures). 1,155×.

FIG. 12.—Cyst-cells in lower end of vas deferens. Zenker-acetic, eosin-Y, and methylene blue. Note extreme vacuolization of cytoplasm and distortion of nuclei (cf. Fig. 2). The apparently darkly stained cyst-cell structures are highly eosinophilic. 436×.

FIG. 13.—Glycogen in cyst-cells, vas deferens. Periodic acid–Schiff. Compare glycogen deposits with empty vacuoles in cells in Fig. 12. 436×.

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# DIURNAL VARIATIONS OF RESPONSE TO LIGHT IN THE FIDDLER CRAB, UCA[1]

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## INTRODUCTION

PERSISTENT diurnal rhythmicity has been studied for a period of more than fifty years, but many aspects of the problem remain almost untouched. The very fact that persistent rhythms have been reported for both plants and animals has perhaps contributed to a tendency to consider each example as a special case. Diurnal rhythms that persist under apparently constant environmental conditions have been described for such widely different groups as the following: Rhythms of germination and growth responsiveness have been reported for fungi (Yarwood and Cohen, 1949). The so-called "sleep movements" of the leaves of angiosperms show, in many cases, a persistent rhythmicity. A considerable body of work has been devoted to studies of this phenomenon, and much of it is reviewed in articles by E. Bünning (1935, 1936). Among crustaceans, locomotor rhythms have been described by Roberts (1936), Kalmus (1938), and Schallek (1942). Diurnal mi-

[1] The author wishes to express her sincere appreciation to Dr. Frank A. Brown, Jr., for his encouragement during the progress of this work and for his helpful criticism of the manuscript.

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grations of retinal pigments in crustaceans were first described by Welsh in 1930. Bennitt (1932) reported a persistent diurnal rhythm of the proximal retinal pigment for crayfish. Further studies in this area are those of Kleinholz (1937*b*), Welsh (1935, 1936, 1941), and Smith (1948). Many crustaceans also exhibit a diurnal rhythm of color change which persists under constant environmental conditions. Kleinholz (1937*a*) described a diurnal rhythm of color change for the Bermudan isopod, *Ligia baudiniana*, and in this paper cites most of the work previously reported, especially that on isopods. Diurnal rhythms of the integumentary chromatophores of decapod crustaceans have been studied most recently by Brown and Webb (1948, 1949). Further examples of diurnal rhythms in animals are the locomotor rhythms in the holothurian, *Thyone briareus* (Stier, 1933), and a great variety of rhythms in the vertebrates.

This list, by no means complete, gives some idea of the extent of occurrence of this phenomenon throughout the plant and animal kingdoms. It is obvious that the mechanisms involved in the immediate control of these varied processes offer no hint of a common factor. However, certain bases for comparison do become evident when one examines a few of the more extensively studied rhythmical processes. An analysis from a comparative viewpoint was performed by Bünning as long ago as 1935, at which time he pointed out certain similarities between diurnal rhythms in insects and in plants. The organisms with which he worked were the bean, *Phaseolus multiflorus*, and the fruit fly, *Drosophila melanogaster*.

*Phaseolus* exhibits a diurnal rhythm of leaf movements which persists under constant conditions. These movements consist of a downward bending of the leaf at night and a corresponding upward movement in the daytime. Bünning, in a series of papers beginning in 1931, has described in considerable detail the characteristics of rhythmicity in this form. The rhythm persists for some time under conditions constant with respect to light —either in constant illumination or in constant darkness. It is possible to reverse the rhythm by illumination at night and darkness in the daytime, and this reversed rhythm then persists under constant conditions. There is a marked dependence upon temperature for maintenance of the frequency. Thus it is only within a small temperature range that the 24-hour period is observed, and an increase of a few degrees results in an increase in the frequency. It is worth noting in this connection that such a dependence upon temperature does not hold for all plants—*Coleus* is reported by Schmitz (1934) to maintain a 24-hour period over a much wider range of temperatures.

Bünning further reports that plants isolated during early stages of development and raised under constant environmental conditions still exhibited diurnal rhythmicity. He also demonstrated that a decrease in the oxygen supply brought an increase in the length of the period (a decrease in frequency) and that a similar effect was obtained by treatment with cyanide.

Turning, now, to the work on *Drosophila*, we find that these insects exhibit a rhythm of emergence from the pupal stage. In a culture of *Drosophila* the emergence of the imagoes tends to occur in the morning, so that a diurnal rhythm is evident. Although the time of emergence can be altered by changes in lighting (Bremer, 1926), a diurnal rhythm persists for some time under constant illumination or constant darkness. The rhythm can be reversed by illumination at night and darkness in the daytime,

and this reversed rhythm then persists for a time under constant conditions. Bünning also obtained a decrease in the frequency of the rhythm when the animals were maintained at a temperature of 10° C. and correspcnding increases in frequency when the cultures were maintained at temperatures higher than normal.

Although *Drosophila* cultures exposed to constant illumination no longer exhibit the diurnal rhythm after a certain length of time, Bünning found that a single light stimulus was sufficient to reestablish the rhythm. He maintained a culture under constant weak illumination through fifteen generations, by which time no rhythm was observable. At the end of this time a single stimulus (brief exposure to darkness) permitted the reappearance of the rhythm of emergence.

It was found that, within the range of oxygen tension at which emergence could occur, the frequency of the rhythm was decreased by a decrease in oxygen supply. Similarly, an enrichment of the atmosphere with oxygen resulted in an increase in the frequency of the emergence rhythm.

The obvious parallels in response to light, temperature, and oxygen were interpreted by Bünning in support of the thesis that endogenous rhythms in plants and animals are, in principle, one and the same phenomenon. Furthermore, he considered that the frequency of the rhythm was genetically determined, at least to the extent that certain genetically imposed limitations existed and that only within these limitations could environmental factors exert their influence. With these conclusions in mind, let us examine some of the other forms of rhythmicity.

An example which at first glance appears to present relatively few parallels to those previously described is the 24-hour framework within which certain learning processes in bees are carried out. This problem has been extensively studied by Beling (1929), Kalmus (1934), and others. It has been found that bees can be trained to approach a feeding station at any time of the day and will thereafter return at the same time each day. Within the temperature range of 18°–32° C. the frequency is unaltered by temperature. With temperatures beyond this range, however, the frequency of the rhythm is definitely decreased by a decrease in temperature. During the test period, food need not be given at the time the bees arrive at the test station; the frequency does not, therefore, seem to depend upon the time required for digestion of food. The frequency of the rhythm can, however, be altered by certain drugs. Quinine, for example, increases the length of the period, while iodothyroglobulin decreases it. The fact that, outside the stated temperature range, this rhythm has a temperature coefficient well within the biological range and the observed effects of the drugs mentioned have led to the conclusion that the observed rhythm is the expression of a basic rhythm of metabolism. Such a conclusion requires the assumption of a temperature-controlling or temperature-compensating mechanism in a poikilothermic organism. While an assumption of this kind appears not to be supported by other physiological studies, it is true that a rather high degree of temperature independence has been described for some other diurnal rhythms. A second assumption which is made in attributing the rhythmicity in training to a basic rhythm of metabolism is that the pharmacology of the drugs mentioned is the same for invertebrates as it is for the higher vertebrates. This is particularly questionable, in view of the inability to demonstrate an effect of iodothyroglobulin upon the metabolism of even some of the lower vertebrates.

Granting the second assumption, then, we find that for bees, as for fruit flies and for beans, the rate at which metabolic reactions proceed within the organism is a factor important in determining the frequency of the rhythm.

Further evidence for a high degree of internal control of rhythmicity is provided by the work of Park and Keller (1932). These investigators reported that the beetle, *Boletotherus cornutus*, exhibits a diurnal rhythm of activity even after being raised from the larval stage under conditions of constant darkness in the laboratory. The extent of tissue reorganization undergone by these organisms between larval and adult stages makes it difficult to interpret these findings in terms of a rhythm impressed upon individual larvae and carried over into adulthood. It is, of course, possible to postulate some environmental factor other than light, temperature, or humidity as the source of maintenance for such a rhythm. Such a postulate is, by its very nature, impossible to disprove; it could only be proved. There are, however, certain characteristics of rhythms which render less probable a dependence upon external factors for maintenance.

One of the environmental factors most commonly effective in bringing about alterations in a rhythm is light. The characteristics of response to light, then, offer a further basis for comparison of various rhythmical processes. Smith (1948) described a persistent diurnal rhythm of retinal pigment migration for three species of grapsoid crabs. This rhythm persisted under conditions of constant darkness for at least 10 days but was not observable in constant illumination, the retinal pigment remaining continuously in the light-adapted condition. It was observed, however, that when animals which had been subjected to constant illumination were placed in darkness at 7:00 A.M. a rhythm was re-established which was out of phase with the normal. The possible significance of this finding will be discussed later.

In 1941 Bentley, Gunn, and Ewer described a diurnal rhythm of activity for the beetle, *Ptinus tectus*. This rhythm persisted for a few days in constant light and then gradually disappeared. When the animals were raised under conditions of constant illumination, no activity rhythm was apparent, but a single period of darkness appeared to be sufficient to permit the establishment of a rhythm which then persisted for a few days in constant light.

Yet another diurnal rhythm whose characteristics of light sensitivity have been studied under conditions which permit comparison with the two just described is that of the daily color change in the fiddler crab, *Uca*. Brown and Webb (1949) found that the chromatophoric expression of the rhythm was gradually inhibited by constant illumination. They also found that the time of occurrence of a given phase of the rhythm which was re-established on return to constant darkness could be varied, depending upon the time at which the animals were placed in the dark. They concluded that a basic center of rhythmicity continued to be active in a rhythmical manner even in the absence of chromatophoric expression of such rhythm and that the basic center was sensitive to the change from light to dark only at certain times.

The distinction between the rhythmical activity of a basic center and the observed expression of the rhythm immediately suggests a similarity among the three cases just described. It will be recalled that the light-inhibited grapsoid crabs were subjected to a single light change—from constant light to constant darkness. This change occurred at a time

which, in *Uca*, represents a period of sensitivity to just such a change. The results are exactly what would be predicted if the animals on which Smith worked are assumed to have, like *Uca*, a basic center of rhythmicity uninhibited by constant light. Turning, now, to a consideration of the results obtained with *Ptinus*, we see that the possibility exists here that the seemingly arhythmic animals actually represent no more than the inhibition of a particular expression of the rhythmicity—in this case, activity. The data available are admittedly only suggestive. Nevertheless, it would seem that the study of what might be termed "rhythms of sensitivity" is likely to produce information relevant to the general problem of rhythmicity.

In this direction some further characteristics of light response of the fiddler crab, *Uca*, have been described by Brown and Webb (1949). Both normal animals and those whose rhythms had been reversed by illumination at night and darkness by day were exposed to 6-hour periods of illumination. Such treatment resulted in a shift of approximately 6 hours in the phases of the rhythm. Upon return to darkness the animals whose rhythms had been normal before the treatment showed a return to a normal rhythm, while those whose rhythm had previously been reversed showed a return to a reversed rhythm. It was concluded that the reversed rhythm showed a degree of stability comparable to that normally found and that, therefore, the basic rhythmicity was actually reversed under conditions of reversed illumination. The basic center was thought not to be affected by these 6-hour periods of illumination, but the fact that the return to normal was only gradual was explained in terms of a second center, capable of limited rhythmic activity, which was gradually brought into phase with the primary center.

In the present work some further characteristics of the response of the basic center to light changes are described. Certain correlations between the sensitivity demonstrated in the laboratory and the maintenance of the rhythm in the animals under the conditions of light changes to which they are normally exposed are considered. And, finally, an attempt will be made to evaluate the results reported here in terms of the general problem of periodicity.

## EXPERIMENTS

All the animals used in these experiments were specimens of *Uca pugnax* collected near Woods Hole, Massachusetts. They were always collected within a day of the beginning of an experiment. For any one experiment ten animals were placed in an aluminum container which had been painted black on the inside. The animals were kept in sea water, just sufficient to cover them, and the water was changed every third day. During periods of darkness the animals were kept in a darkroom and, during periods of illumination, in the laboratory. The source of illumination was a fluorescent light placed directly above the containers. The containers were protected from daylight, so that the intensity of illumination was the same at all times of the day. Unless otherwise stated, the intensity of light used was 90–95 foot-candles as measured by a Weston photometer at the level of the bottom of the containers. For each series of experiments at least one group of ten animals was kept in a container in the darkroom for the duration of the series. The chromatophores of both control and experimental animals were examined at 6-hour intervals throughout the course of an experiment. The times of examination were

1:00 A.M., 7:00 A.M., 1:00 P.M., and 7:00 P.M. The condition of the chromatophores was recorded for each animal in terms of the scale described by Hogben and Slome (1931). The average condition for a group was then determined, so that any one point on a graph represents the average for ten animals. In each of the figures the ordinate gives the degree of dispersion of pigment on the Hogben and Slome scale (stage 1 represents complete concentration; stages 2, 3, and 4, increasing degrees of dispersion; stage 5, complete dispersion). The light and dark segments at the top of the graph and corresponding ones below represent time in days, the blackened segments indicating the interval 6:00 P.M. to 6:00 A.M. and the light segments 6:00 A.M. to 6:00 P.M. The lowermost band represents the experimental treatment; the light areas are periods of illumination and the dark ones periods of darkness. Figure 1 shows the typical diurnal rhythm exhibited in constant darkness.

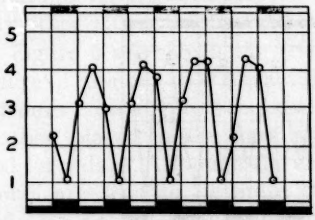


FIG. 1.—The normal diurnal rhythm of the black chromatophores of *Uca* kept in constant darkness. (See text for the interpretation of the figures.)

## SERIES I

### 32-HOUR CYCLES

Figure 2 shows the results obtained when a group of animals was exposed to 16-hour periods of light alternating with 16 hours of darkness. This treatment was continued until 9 successive light periods had been given and was then followed by 7 days in constant darkness. The last period of illumination was from 3:00 P.M. to 7:00 A.M. As can be seen from the graph, when the illumination coincides with normal daytime, the chromato-

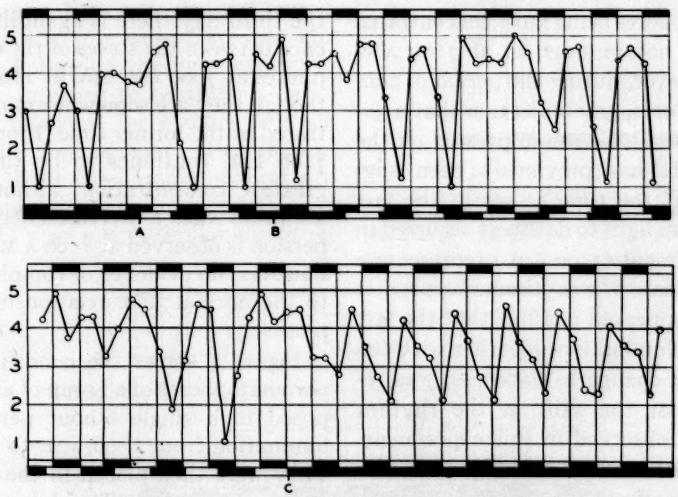


FIG. 2.—Beginning at *A* (11:00 P.M.) *Uca* were exposed to 16-hour periods of illumination alternating with 16-hour periods of darkness. At *B* an illumination period was accidentally omitted. At *C* the animals were returned to constant darkness.

phores appear to have a normal rhythm, with the time of greatest concentration at 1:00 A.M. When, however, the illumination occurs during the night, there is always seen a relatively broad dispersal at 1:00 A.M., and this is not always the time of greatest concentration.

When the animals are placed in constant darkness, there is a continuous increase in degree of concentration during the first 24 hours, with the result that the chromatophores are most concentrated at the next 7:00 A.M. From this point on, there is a 24-hour rhythm, with the chromatophores being most concentrated (although not in stage 1) at 7:00 A.M. There is never, during the period of constant darkness, any indication that a 32-hour rhythm has been impressed on the animals. It has previously been suggested that the time at which the last change from light to darkness occurred in this experiment (7:00 A.M.) represents a sensitive phase of a rhythmical center. It therefore appeared possible that the last period of illumination, together with the subsequent change to darkness, was responsible for the shift in the rhythm which was observed in this experiment. Experiments were then designed to determine the effects of a change from light to darkness at 7:00 A.M. following light-exposure periods of different durations.

SERIES II

LIGHT PERIODS ENDING AT 7:00 A.M.

Figure 3 represents the chromatophore condition of a group of animals illuminated repeatedly from 1:00 A.M. to 7:00 A.M. After five such periods of illumination they were placed in continuous darkness and observed for 5 days. On the first day of observation the chromatophores are seen to be more concentrated than stage 2 at both 7:00 P.M. and 1:00 A.M. On the second day they are at about stage 1 at 7:00 P.M. and are quite broadly dispersed at all other times. On the third day there is again little difference between the states of the chromatophores at 7:00 P.M. and at 1:00 A.M., although they are somewhat more concentrated at the former time. From here on, 7:00 P.M. continues to be the time of greatest concentration, although there are times when a decreased degree of dispersion is observed at 1:00 A.M. There is, however, no evidence of complete return to the normal time relationships of the phases.

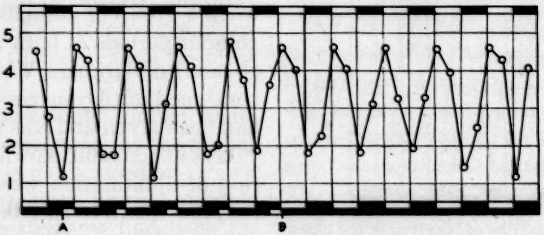


FIG. 3.—*Uca*, otherwise in constant darkness, were exposed daily to 6-hour periods of illumination (1:00 A.M. to 7:00 A.M.), beginning at *A*. At *B* they were returned to constant darkness.

Figure 4 shows the condition of the chromatophores of a group of animals exposed to a single 6-hour period of illumination from 1:00 A.M. to 7:00 A.M. They were then placed in the dark and observed for 5 days. The chromatophores are seen to be almost completely concentrated at 7:00 P.M. and at 1:00 A.M. after

the light exposure. At the other times of observation (7:00 A.M. and 1:00 P.M.) the pigment is broadly dispersed.

Figure 5 represents the chromatophoric condition of a group of animals placed in the dark at 1:00 P.M. and remaining in darkness until the fourth day, when they were illuminated for 6 hours, from 1:00 A.M. to 7:00 A.M. They were then returned to darkness and observed during the next 3 days. There appears to be no change in the rhythm of the chromatophores. The only irregularity is that at the end of the period of illumination the chromatophores are more dispersed than at any other 7:00 A.M. observation.

In Figure 6 are shown the results of exposure of animals to a single light period from 11:00 A.M. to 7:00 A.M. They were then placed in the dark and observed for 3 days. Although the chromatophores are seen to be more concentrated at 7:00 A.M. than under normal conditions, the time of greatest concentration is still at 1:00 A.M.

The results of exposure of a second group of animals to a single light period from 11:00 A.M. to 7:00 A.M. are found in Figure 7. Following the light exposure, they were returned to continuous darkness and observed for 6 days. We see that in the first 24 hours after the exposure to light the chromatophores are almost completely concentrated at 1:00 A.M. and at 7:00 A.M. A similar condition

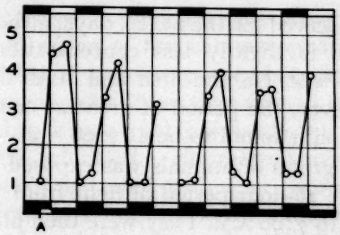


FIG. 4.—*Uca*, beginning at *A*, were exposed to a single 6-hour period of illumination. They were otherwise kept in constant darkness.

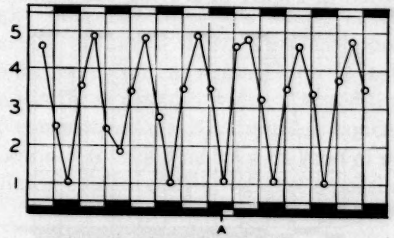


FIG. 5.—*Uca*, otherwise in constant darkness, were exposed to a single 6-hour period of illumination (1:00 A.M. to 7:00 A.M.), beginning at *A*.

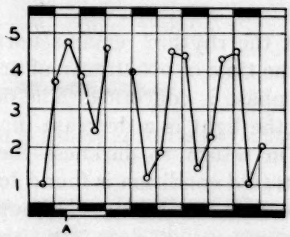


FIG. 6.—*Uca*, otherwise in constant darkness, were exposed to a single 20-hour period of illumination (11:00 A.M. to 7:00 A.M.), beginning at *A*.

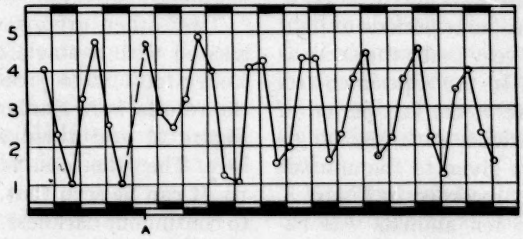


FIG. 7.—*Uca*, otherwise in constant darkness, were exposed to a single 20-hour period of illumination (11:00 A.M. to 7:00 A.M.), beginning at *A*.

is observed for the next 3 days, although there is slightly less concentration at 7:00 A.M. On the fifth and sixth days, however, the period of greatest concentration is found to be at 7:00 A.M.

A group of animals was exposed to a single 16-hour period of light from 3:00 P.M. to 7:00 A.M. They were then placed in the dark and observed until the fifth day. The conditions of the chromatophores during the experiment are recorded in Figure 8. During the exposure

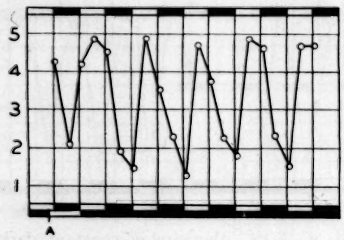


FIG. 8.—*Uca*, otherwise in constant darkness, were exposed to a single 16-hour period of illumination (3:00 P.M. to 7:00 A.M.), beginning at *A*.

to light the rhythm remains normal so far as the time of occurrence of any particular phase is concerned. The only effect of the light is a decrease in amplitude. On return to darkness the most concentrated condition is found to occur at 7:00 A.M. each day, although the chromatophores at 1:00 A.M. are only slightly more dispersed than stage 2. It will be recalled that the conditions shown in Figure 2, when the animals were placed in continuous darkness after regularly alternating 16-hour periods of light and darkness, are very much the same as those found here. In the experiment referred to in Figure 2, the last period of illumination coincided with the single period of exposure given to the animals whose condition is recorded in Figure 8. Another group of ten animals was exposed to a single period of light from 3:00 P.M. to 7:00 A.M., with results essentially the same as those seen in Figure 8. Because of this similarity, a graphic representation of the results is not included.

Figure 9 shows the results of a single 12-hour period of exposure to illumination. A group of ten animals was placed in the dark at 7:00 P.M.; after 24 hours they were exposed to light from 7:00 P.M. to 7:00 A.M. They were then returned to continuous darkness. It can be seen that during the time of illumination the chromatophores are more dispersed than they are at such a time under normal conditions. After return to darkness, the first observation (1:00 P.M.) shows the chromatophores to be more concentrated than at the preceding time of recording. There is, however, no permanent effect on the rhythm, as is seen from the conditions recorded for the next 48-hour period. During this time the most concentrated condition occurs regularly at 1:00 A.M., at which time the chromatophores are in stage 1.

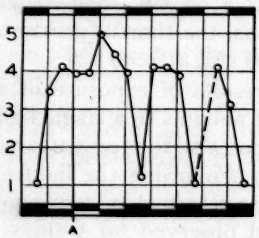


FIG. 9.—*Uca*, otherwise in constant darkness, were exposed to a single 12-hour period of illumination (7:00 P.M. to 7:00 A.M.), beginning at *A*.

Two other groups of animals were treated with a single exposure to light from 7:00 P.M. to 7:00 A.M. In one case the results were similar to those seen in Figure 9, and these are not included here. The other case is shown in Figure 10. It can be seen that, following return to continuous darkness, the most concentrated condition of the chromatophores occurs daily at 1 A.M., although they are

only slightly more dispersed at 7:00 A.M.

Figure 11 represents the condition of the chromatophores of animals which were exposed to light for a period of 24 hours from 7:00 A.M. to 7:00 A.M. They were then returned to continuous darkness and observed for 4 days. In the first

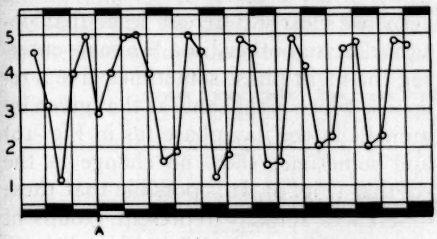


FIG. 10.—*Uca*, otherwise in constant darkness, were exposed to a single 12-hour period of illumination (7:00 P.M. to 7:00 A.M.), beginning at *A*.

those cases in which almost complete concentration of the chromatophores is recorded at two successive observations represent shifts of less than 6 hours in the rhythm or an actual increase in the time during which the chromatophores remain concentrated, with a corresponding decrease in time of dispersal. Either condition would result in data similar to those obtained, since observations were made at 6-hour intervals.

The assumption that stimulation by light does not alter the shape of the curves obtained when chromatophore condition is plotted against time is justified in those cases in which a single time of complete or nearly complete concentration is found. The only method of obtaining evidence for or against a possible

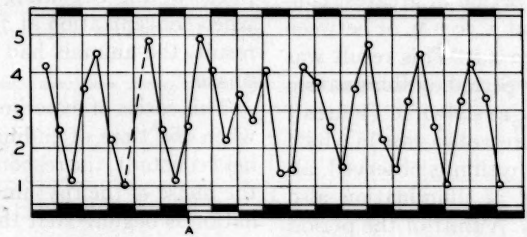


FIG. 11.—*Uca*, otherwise in constant darkness, were exposed to a single 24-hour period of illumination (7:00 A.M. to 7:00 A.M.), beginning at *A*.

24 hours after exposure to light the chromatophores are almost completely concentrated at 1:00 A.M. and are in the same state at 7:00 A.M. On the second day and every day thereafter the most concentrated condition occurs at 7:00 A.M.

Although further experiments dealing with 12-hour periods of illumination are to be described later, it would be well to consider here possible interpretations of the results recorded in Figures 3–11, inclusive.

## DISCUSSION

The first question to arise in a discussion of these experiments is whether increase in the length of time during which the chromatophores remain concentrated would be by observing the chromatophores at more frequent intervals. This has not been done. Therefore, in the absence of evidence concerning alterations in the shape of the curve, we shall assume that only one kind of change in rhythm occurs, namely, the shift in time, with all phases remaining proportionately the same length as in the normal.

With respect to the condition of the chromatophores at the beginning of the illumination periods, the experiments recorded in Figures 3–11 fall into four

groups. The pigment may be completely concentrated when the illumination begins—this is true of those experiments in which illumination began at 1:00 A.M. The chromatophores may be just beginning to concentrate, and this condition is shown in those experiments in which illumination began at 7:00 P.M. The chromatophores may be in the day phase (completely dispersed); such a condition occurs when illumination is begun at 3:00 P.M. or 11:00 A.M. Or, finally, the chromatophores may be just beginning to disperse, and this is true when the period of illumination begins at 7:00 A.M. When the results obtained by illumination under these four different conditions are examined, it is seen that they fall into roughly three groups.

*Group 1.*—The period of greatest concentration occurs at 7:00 P.M. or between 7:00 P.M. and 1:00 A.M. This result was obtained when the period of illumination began at 1:00 A.M., as shown in Figures 3 and 4. Figure 5 represents a case in which no change in the rhythm is observed, although the period of illumination also begins at 1:00 A.M. A shift of the period of greatest concentration toward 7:00 P.M. was obtained under no other conditions.

*Group 2.*—There may be no observed change in the rhythm. This was found to be true in two of the cases in which animals were exposed to a single light period from 7:00 P.M. to 7:00 A.M. (Fig. 9).

*Group 3.*—The period of greatest concentration occurs at 7:00 A.M. or between 1:00 A.M. and 7:00 A.M. Results of this kind were obtained when the periods of illumination were 16, 20, or 24 hours long (Figs. 6, 7, 8, and 11). A similar result was also obtained in one case of a 12-hour period of illumination from 7:00 P.M. to 7:00 A.M.

This classification of results parallels the classification on the basis of the condition of the chromatophores at the beginning of illumination, except that one of these latter groups drops out, since the response of animals entering the day phase is the same as that of animals already in it. The animals in the night phase when illumination begins give a response clearly different from that obtained at any other time. Animals entering the night phase sometimes give a response which is the same as that given by animals in the day phase (as in Fig. 10) and sometimes show no change in the rhythm (Fig. 9). It is possible that these differences actually represent groups of animals which are in different conditions: when no change occurred, the animals were in transition from day to night phase at the beginning of illumination; when concentration at 7:00 A.M. was the result, the animals had been in the day phase.

This series of experiments shows that, when the time of ending illumination is held constant, the response depends upon the phase of the rhythm at which illumination is begun. That this factor is more important than the actual length of the period of illumination is seen from the fact that periods of illumination lasting 16, 20, or 24 hours all resulted in the same response.

Another observation of importance is that whenever a shift in rhythm is effected in this series of experiments the shift persists in constant darkness. This fact tends to support the interpretation that it is possible to alter the rhythm through one of two pathways, one resulting in a shift of a basic center of rhythmicity; and this is the center which is affected by a change from light to dark at or near 7:00 A.M. The other pathway would involve a center capable of limited rhythmicity and is ultimately brought under control of the basic center. The

effects of this postulated second center are not encountered in this particular series.

### SERIES III

#### REVERSED ILLUMINATION

A series of experiments will now be described in all of which the period of illumination lasted from 7:00 P.M. to 7:00 A.M. but the periods were repeated on successive days until a reversal of the

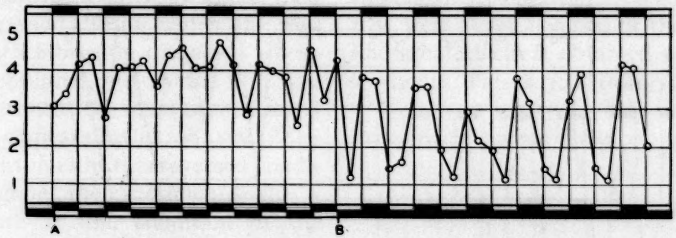


FIG. 12.—*Uca*, beginning at *A*, illuminated at night (7:00 P.M. to 7:00 A.M.) and kept in darkness in the daytime. At *B* they were placed in constant darkness.

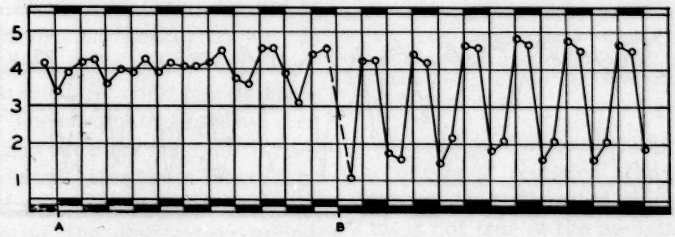


FIG. 13.—*Uca*, beginning at *A*, were illuminated at night (7:00 P.M. to 7:00 A.M.) and kept in darkness during the day. At *B* they were returned to constant darkness.

observed chromatophoric rhythm was obtained.

Figure 12 shows the condition of the chromatophores when animals were illuminated from 7:00 P.M. to 7:00 A.M. on 6 successive days and were then placed in constant darkness for 6 more days. In the first 24 hours of the experiment the most concentrated condition of the chromatophores is found at 7:00 P.M. On the second day a similar observation is made, although here the degree of concentration is considerably less. By the third day there is no single time at which the pigment is significantly more concentrated than at any other time, but by the fourth day 1:00 P.M. is clearly such a time. There is then no further change in phase while regular exposure to light continues. An increase in amplitude is found, so that during the first 24 hours in darkness the chromatophores are in stage 1 at 1:00 P.M. and are close to stage 4 at 1:00 A.M., 7:00 A.M., and 7:00 P.M. The following day the most concentrated condition occurs at 7:00 A.M. From this time until the experiment is concluded the chromatophores are never more dispersed than stage 2 at 7:00 A.M., but they are more concentrated at 1:00 P.M.

Figure 13 represents the condition of the chromatophores of another group of animals exposed on successive days to illumination from 7:00 P.M. to 7:00 A.M. Coinciding with the first three regularly repeated exposures, there is a gradual

disappearance of the rhythmical activity, so that in the fourth dark period the chromatophores remain broadly dispersed. By the sixth dark period there is distinctly more concentration observed at 1:00 P.M. than at other times. In the following 24 hours it is found that the chromatophores are most dispersed at 1:00 A.M. and most concentrated at 1:00 P.M., which is a complete reversal of

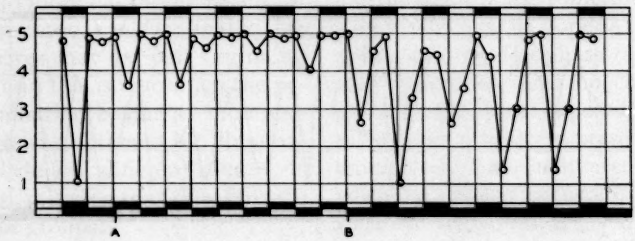


FIG. 14.—*Uca*, beginning at *A*, were illuminated at night (7:00 P.M. to 7:00 A.M.) and kept in darkness during the day. At *B* they were returned to constant darkness.

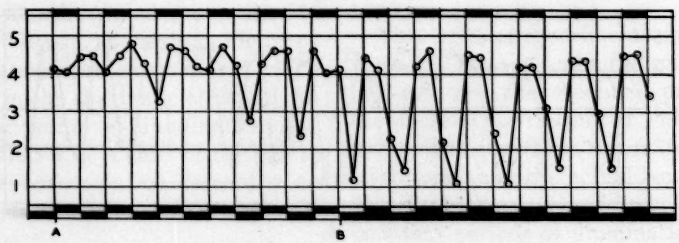


FIG. 15.—*Uca*, beginning at *A*, were illuminated at night (7:00 PM.. to 7:00 A.M.) and kept in darkness during the day. At *B* they were returned to constant darkness.

the normal condition. On the first day in continuous darkness there is little difference between the state of the chromatophores at 7:00 A.M. and that observed at 1:00 P.M., the pigment being almost completely concentrated at both times. After this day it is found to be most concentrated at 7:00 A.M., and this situation persists until the end of the experiment.

Another group of animals was exposed to illumination by night and to darkness by day. This exposure period (7:00 P.M. to 7:00 A.M.) was repeated five times, after which the animals were placed in constant darkness for 4 days. The record of the states of the chromatophores throughout the experiment is presented in Figure 14. During the first 2 days of this experiment, the chromatophores, while quite broadly dispersed at all times, give definite evidence of concentration at 1:00 A.M.—the normal time. On the third day there is no time at which any marked degree of concentration is seen. On the fourth and fifth days concentration is greatest at 1:00 P.M. During the first 24 hours in the dark there is a period of concentration recorded at 1:00 P.M., followed by dispersal; but by 7:00 A.M. the chromatophores are in stage 1. From this time on, the most concentrated condition is seen to occur at 7:00 A.M. each day.

In Figure 15 are recorded the condition of the chromatophores of another group of animals under conditions of reversed illumination and subsequent main-

tenance in constant darkness. The times of exposure to light were from 7:00 P.M. to 7:00 A.M., and the intensity of illumination was 200 foot-candles. The periods of exposure were continued for 6 days; then the animals were placed in the dark for another 6 days. It can be seen that in the second 24-hour period of alternating light and darkness the most concentrated condition of the chromatophores occurs at 7:00 P.M. There then follows a period during which the pigment is rather broadly dispersed whenever it is observed, but by the fifth day of the experiment the most concentrated condition appears at 1:00 P.M. The rhythm continues in this reversed condition throughout the experiment.

In Figure 16 the condition of the chromatophores is recorded for a group of animals being maintained in constant darkness after they had previously been exposed to illumination at night and to darkness in the daytime (7:00 P.M. to 7:00 A.M.) for a period of 1 week. They were maintained in constant darkness for 6 days. The chromatophores are seen to be in stage 1 each day and quite broadly dispersed at all other times. There is no evidence of any change in phase of the rhythm during the observation period.

## DISCUSSION

Figures 12–16, then, represent the results obtained when different groups of animals were exposed to illumination at night and to darkness in the daytime. In the past this experimental procedure has been found to result consistently in a reversal of the rhythm, and such a reversed rhythm has been shown to persist for at least a week. It is therefore surprising to find that the end-results obtained here can be arranged in a graded series, from complete reversal to a 6-hour shift, with the most concentrated condition of the chromatophores occurring at 7:00 A.M. This is true in spite of the fact that reversal had occurred in all cases before the animals were placed in constant darkness. There appears to be no corresponding variations in either the external experimental conditions or in the observed conditions of the chromatophores during the course of reversal.

It was seen in a previous series of experiments that the results of illumination for a single period from 7:00 P.M. to 7:00 A. M. were never reversal of the rhythm. The greatest effect obtained was a shift of 6 hours. This period (from 7:00 P.M. to 7:00 A.M.) is unique, therefore, in that the effects of repeated stimulation are different from those of single periods. This is not true of the periods 7:00 P.M. to 1:00 A.M. or 1:00 A.M. to 7:00 A.M., for example. The possibility is suggested that the reason for the requirement of repeated periods to obtain reversal is that the total process is accomplished by abrupt shifts of not more than 6 hours each. It will be recalled that in those experiments utilizing single light periods it was not unusual to obtain results which could be interpreted as showing a shift of somewhat less than 6 hours. On the other hand, no single shift in rhythm of more than 6 hours has been observed. If, therefore, the first 12-hour period is considered to have brought about a shift of less than 6 hours, we can see that a subse-

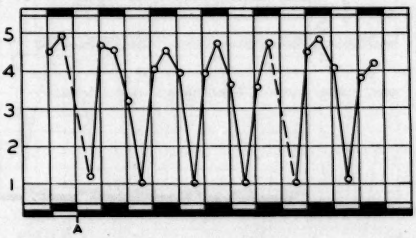


FIG. 16.—*Uca*, having previously been exposed to illumination at night (7:00 P.M. to 7:00 A.M.) and darkness in the daytime, were returned to constant darkness at *A*.

quent shift, resulting from a repetition of this 12-hour period, would not result in the basic center becoming precisely 12 hours out of phase with the normal rhythm. This would be equally true, of course, if it were the second period of illumination which effected a shift of less than 6 hours instead of the first period. It is quite possible that either a direct response of the chromatophores to light or a secondary center of rhythmicity, more directly influenced by light, would prevent this condition from being observed until the animals had been placed in the dark. The latter factor seems more likely, in view of the fact that it sometimes requires a few days for the final condition of the rhythm to become established in continuous darkness.

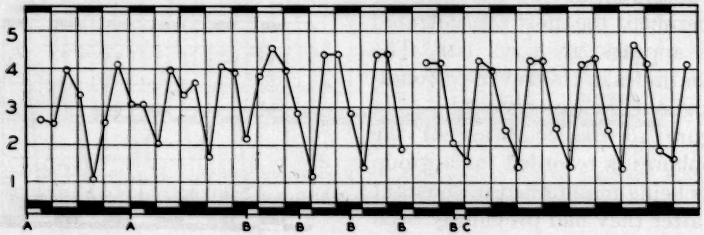


FIG. 17.—*Uca*, at the two times indicated by *A*, were exposed to 6-hour periods of illumination (7:00 P.M. to 1:00 A.M.), and at the four times indicated by *B* they were exposed to 6-hour periods of illumination (1:00 A.M. to 7:00 A.M.). At *C* they were returned to constant darkness.

If it is true that the reversal of the rhythmicity usually observed following repeated exposures to illumination by night and darkness by day is accomplished by successive shifts of 6 hours or less in the rhythmical center, then it should be possible to effect a reversal of the rhythm by appropriate 6-hour periods of illumination. The following experiments were designed to test the feasibility of such a procedure.

## SERIES IV

### 6-HOUR ILLUMINATION PERIODS

A group of ten animals was exposed to illumination for a period of 6 hours from 7:00 P.M. to 1:00 A.M. For the next 42 hours they were in darkness. After this, again at 7:00 P.M., a second 6-hour period of illumination was given. There followed a 48-hour period of darkness, after which the animals were illuminated from 1:00 A.M. to 7:00 A.M. daily for 4 days. They were then left in darkness for the remainder of the experiment—a period of 6 days. The condition of the chromatophores throughout the experimental period is recorded in Figure 17. It can be seen that at 1:00 A.M. following the first illumination period the chromatophores are more dispersed than they are at this time under normal conditions. The next 1:00 A.M. observation shows the chromatophores in stage 1. At the end of the second illumination period (1:00 A.M.), the chromatophores are in stage 3, and they become more concentrated thereafter, so that at 7:00 A.M. they are in stage 2. Although some irregularities are observed during the next 24 hours, the chromatophores are clearly more concentrated at 7:00 A.M. than at any other time. There is some evidence that this displaced rhythm is returning to normal when the illumination periods from 1:00 A.M. to 7:00 A.M. are initiated. This is suggested by the fact that the chromatophores are more concentrated at the beginning of the first such period then they are at the preceding 1:00 A.M. At 7:00 A.M. they are broadly dispersed (stage 4) and are not

completely concentrated again until the next 7:00 A.M. From then until the end of the experiment there is a 24-hour cycle, with the most concentrated condition of the chromatophores occurring at 7:00 A.M. each day.

In Figure 18 is found the record of a group of ten animals exposed to light from 1:00 A.M. to 7:00 A.M. daily for 3 days. For the next 5 days they were kept in darkness and then were exposed to light from 7:00 P.M. to 1:00 A.M. on 3 successive days. Following this treatment they were in continuous darkness for 4 days. After the first 6-hour period of illumination the chromatophores are seen to be more concentrated at 7:00 P.M. than they are under normal conditions, but the most concentrated condition is still found at 1:00 A.M. After the second exposure to light and continuing through the 5-day period of darkness, the most concentrated state of the chromatophores occurs at 7:00 P.M. During the 3 days on which the animals were illuminated from 7:00 P.M. to 1:00 A.M. there appears to be a gradual shift until, after the third light exposure, the chromatophores show a rhythm of good amplitude, with the phase of concentration occurring at 1:00 P.M. During the 4 days in continuous darkness there is no evidence of any further alteration. The final condition is, therefore, a reversed rhythm which has persisted in constant darkness for 4 days.

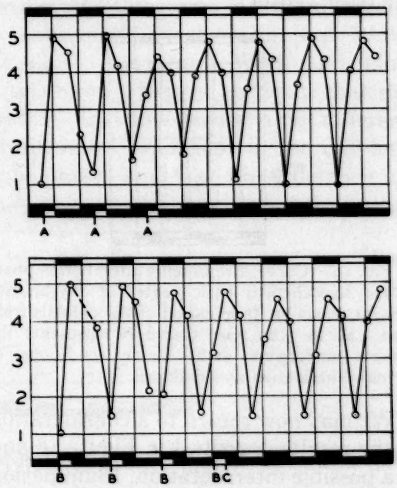


FIG. 18.—*Uca*, otherwise kept in darkness, were exposed to 6-hour periods of illumination (1:00 A.M. to 7:00 A.M.) at the three times indicated by *A*. At the four times indicated by *B* they were exposed to 6-hour periods of illumination (7:00 P.M. to 1:00 A.M.). At *C* they were returned to constant darkness.

## DISCUSSION

In any reasonable interpretation of the observations recorded in Figures 17 and 18 certain other observations must be borne in mind. These may be briefly summarized: (1) A permanent reversal of the rhythmical activity may be obtained by repeatedly illuminating from 7:00 P.M. to 7:00 A.M. (2) Such a reversal is not achieved by a single illumination period. (3) Illumination from 7:00 P.M. to 1:00 A.M. results in a shift in the observed rhythm such that the most concentrated period is recorded at 7:00 A.M., but this shift is only temporary. (4) Under certain conditions it is possible to obtain a shift of the rhythm so that the most concentrated condition of the chromatophores occurs at 7:00 A.M. by a single illumination period from 7:00 P.M. to 7:00 A.M. When this result is obtained, the shift in the rhythm is permanent. (5) Illumination from 1:00 A.M. to 7:00 A.M. results in a shift in the rhythm such that the most concentrated condition of the chromatophores is found at 7:00 P.M., and this condition persists. It seems obvious that the observations summarized under points 3 and 4 above must be accounted for in terms of two different operative mechanisms. The requisite mechanisms are provided by the hypothesis that a basic center of rhythmic-

ity is sensitive to a change from light to dark at a definite phase of its cycle. This sensitive phase occurs around 7:00 A.M. in a normal rhythm. If, however, the change from light to dark occurs at some other time, a secondary center may be affected, but this secondary center will eventually be brought into phase with the basic center.

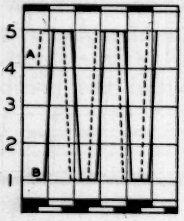


FIG. 19.—Curve *A* represents alteration in phase of the hypothetical basic center of rhythmicity brought about by illumination of the animals from 1:00 A.M. to 1:00 P.M. Curve *B* represents the changes undergone daily under conditions of normal illumination and darkness.

We may now return to a consideration of the results described in Figure 18 and to a possible interpretation. Illumination from 1:00 A.M. to 7:00 A.M. results in dispersion and concentration occurring 6 hours earlier than normal, and this shift is permanent because a corresponding alteration in the rhythm of the basic center has occurred. If this is true, then the subsequent illumination from 7:00 P.M. to 1:00 A.M. occurred at a time in the cycle corresponding to 1:00 A.M. to 7:00 A.M. The relationships between the phases of the rhythm at the time recorded, on the one hand, and "physiological time," on the other, are shown in Figure 19. The results obtained are what would be expected if this is a true description. The rhythm is shifted in such a manner that corresponding phases occur 6 hours earlier than they did after the first illumination periods, and the shift is permanent. The end-result, then, is that the most concentrated condition of the chromatophores occurs at 1:00 P.M.—that is, the rhythm has been reversed.

Figure 20 represents the relationships between the rhythm of the basic center and that of the chromatophores as they may exist in the experiment described in Figure 17, following the first illumination. It seems probable that the basic center in this case remains in phase with the normal and that, therefore, the second period of illumination occurred at a time in the cycle which corresponds precisely to the stated time (1:00 A.M. to 7:00 A.M.). Such a condition would lead us to predict that the chromatophoric rhythm resulting from the second illumination period would exhibit the period of greatest concentration at 7:00 P.M. Instead we find the period of greatest concentration at 7:00 A.M. Furthermore, this shift in rhythm is persistent,

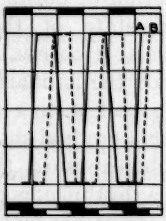


FIG. 20.—Curve *A* represents the daily changes undergone by the hypothetical basic center of rhythmicity either under normal conditions of illumination or under conditions such that animals are illuminated from 1:00 P.M. to 1:00 A.M. and kept in darkness during the rest of the day. Curve *B* represents diagrammatically the rhythmical changes observed in the chromatophores when the animals have been illuminated from 1:00 P.M. to 1:00 A.M. and kept in darkness during the remainder of the day.

and thus it must be interpreted as a shift in the basic rhythm. It will be noticed that the result obtained here corresponds to that consistently found when animals are exposed to an illumination period which begins in the day phase and ends at 7:00 A.M. It must be obvious that an observed response by the center to the

stimulus provided by light changes depends not only upon the excitability of the center but also upon the ability of the appropriate sense-receptors of the organism to receive the stimulus. Although it has been demonstrated that, regardless of the actual condition of the chromatophores at 7:00 A.M., a change from light to dark at that time constitutes an adequate stimulus, it has not been shown that it is also true that, regardless of the chromatophoric conditions, the change from dark to light is always effective at 1:00 A.M. The chromatophoric rhythm quite likely is only one of several processes whose rhythmicity is ultimately controlled by the basic center; so it is not necessary to conclude that the condition of the chromatophores in itself is a limiting factor in the effectiveness of the stimulus of dark-to-light changes. Further experiments must, of course, be performed before any definite conclusions can be drawn, but the results recorded in Figure 17 at least raise a question concerning the conditions under which the change from darkness to light is an effective stimulus to the center.

In this connection it may be pointed out that two rhythms have been described in the literature which might well have some bearing upon a change in the response to light observed at a given phase of the diurnal rhythm. Rhythms of retinal pigments have been mentioned previously in this paper, and it seems possible that a temporary shift in these rhythms might involve a pigment position such that the normal nighttime response to light would not occur. Another diurnal rhythm which offers interesting possibilities in this respect is that described by Jahn and Crescitelli (1940) for the electroretinograms of certain insects. In subsequent work Jahn and Wulff (1941*a*, *b*, 1943) and Wulff and Jahn (1943) have presented evidence that the rhythm involves essentially different physiological conditions in the day and night phases and that the difference includes rhythmical alterations in sensitivity.

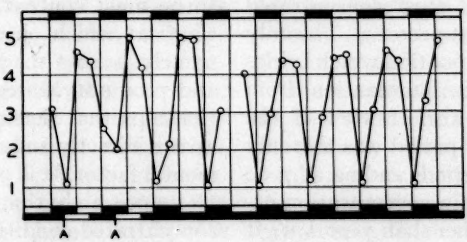


FIG. 21.—*Uca*, otherwise in constant darkness, were exposed to 12-hour periods of illumination (1:00 A.M. to 1:00 P.M.) at the two times indicated by *A*.

## SERIES V

### LIGHT PERIOD BEGINNING AT 1:00 A.M.

Some of the experiments described earlier in this paper showed that, when the time of ending a period of illumination was constant (at 7:00 A.M.), the actual duration of the period was unimportant as compared with the phase of the rhythm at which illumination was begun. The results recorded in Figure 21 enable one to compare similarly the effects of different durations of light when the time of beginning of illumination is held constant. Beginning at 1:00 A.M. the animals were exposed to a 12-hour period of illumination. Twenty-four hours later a second 12-hour period of light was begun.

At the end of this second period the animals were placed in darkness for 5 days. It is seen that for the first 24 hours the only change is that the degree of concentration at 1:00 A.M. is less than that found under normal conditions of illumination. After the second period of illumination the chromatophores are found to be in stage 1 at 7:00 P.M. and to be more dispersed at 1:00 A.M. For the next 5 days, in continuous darkness, the rhythm continues with a 24-hour cycle, with the most concentrated condition occurring at 7:00 P.M. This experiment is one of the few that resulted in a 6-hour shift such that the most concentrated condition occurred at 7:00 P.M. The only other period of illumination which brings about this result is illumination from 1:00 A.M. to 7:00 A.M. In view of the fact that this latter period was the only one in a series of periods ending at 7:00 A.M. which resulted in concentration occurring at a time other than 7:00 A.M., it appears that, under these conditions, the time of beginning the illumination period is the factor determining the direction of the shift in the rhythm.

## DISCUSSION

A consideration of those experiments in which the time of either beginning or ending the period of illumination was held constant while the duration of the periods varied leads to the conclusion that the basic center of rhythmicity is sensitive to light changes under two conditions. During the night phase the change from dark to light brings about a response, and the response can be correlated more nearly with the time at which the change occurs than with the duration of the stimulus. During the transition to the day phase, the basic center responds to a change from light to darkness, and the response appears to be independent of the duration of the preceding light period, provided only that a certain minimum duration is attained. The minimum duration probably is simply that the exposure to light shall have begun during the day phase—that is, in a nonsensitive time.

## GENERAL DISCUSSION

A consideration of all the evidence available reveals that, within the limits of the 24-hour cycle, the time of occurrence of any particular phase is controlled within the organism. Thus, persistent rhythms have been demonstrated in which the chromatophores are found to be most concentrated at any of the times at which observations are made, namely, 1:00 A.M., 7:00 A.M., 1:00 P.M., and 7:00 P.M. Moreover, the phase relationships that persist need not be such as are directly induced by an environmental factor. It is possible, for example, to induce a rhythm in which the most concentrated condition of the chromatophores occurs at 7:00 A.M. and which, upon return to constant darkness, exhibits a shift until the most concentrated condition occurs at 1:00 A.M. It is likewise possible to induce a rhythm showing the most concentrated condition at 1:00 P.M.; and, under certain conditions, return to darkness is followed by a gradual shift to a condition in which the most concentrated state is at 7:00 A.M. There appears, then, to be a center of rhythmicity within the organism which exerts a high degree of control over the phases of observed rhythmical processes.

Although this center can be influenced by light, it can continue to act rhythmically in the absence of light for long periods of time. This is shown by the persistence of rhythmic activity in continuous darkness. We know, too, that continuous illumination does not inhibit the center itself. This has been demonstrated by those experiments in which the chro-

matophoric expression of the rhythm is inhibited by prolonged illumination and the animals are then returned to darkness. The time of change from light to dark determines the response—that is, the sensitivity is still rhythmical.

On the other hand, the center has been shown to have its activity altered by certain light changes. Thus the change from dark to light during the night phase and the reverse change from light to dark during the transition from night to day phases are both effective in altering the rhythmical center. It appears also that, having once evoked the response to the change from dark to light, it becomes impossible to obtain, within the day phase thus produced, subsequent response to the change from light to dark. This is explained logically by the assumptions that the response to a nighttime change from dark to light is that the center actually does enter the day phase and that in the day phase there is no response by the center to any light changes.

The center thus appears to be sensitive to light during the night phase and to the change from light to darkness during the transition from night to day phases. These variations in sensitivity are of significance in relation to the possession of a rhythm by these animals under natural conditions. When it is realized that the crabs enter their burrows at high tide, it becomes obvious that they are exposed daily to light changes other than those involved in day and night. For example, if high tide occurs on a certain day at 3:00 P.M., the animals have been exposed to daytime illumination and have gone into darkness sometime around noon, but the center of rhythmicity, being in the day phase, is not sensitive to light changes. On such a day, low tide would be around 9:00 P.M. If the animals emerge from their burrows before dark, they will still undergo the normal change from light to dark. If they do not emerge until after dark, it is of no consequence—the rhythm persists in continuous darkness. A few days later, a high tide would occur in the morning at such a time that they might enter their burrows in daylight and possibly thus undergo the change from light to darkness at or near 7:00 A.M. But, if this is to occur, they must first have been exposed to light, and the original change from dark to light would be the effective stimulus. It is thus that the observed variations in sensitivity readily explain how it is possible for these animals to show considerable sensitivity to light changes, to be frequently exposed to such light changes, and to exhibit a rhythm of such constancy.

There are, in general, three types of approach to the study of the problem of rhythmicity. The first of these types would provide further details concerning the characteristics of the basic rhythmical process. From this type of approach we might expect to learn to what extent the characteristics are similar in all organisms. Eventually we would expect to be able to identify the reaction or series of reactions responsible for the frequency of the rhythm. In the second type of approach one would obtain more complete descriptions of the mechanisms involved in control of what may be termed the "indicator activities." These are the activities which exhibit a rhythmicity normally controlled by a basic center but which are also capable of being influenced to a certain extent by factors other than the basic rhythmicity. The third type of approach would consider the significance of rhythmicity to the organism. Does it provide a relatively stable background upon which are built certain long-range responses? For example, it has been shown that the day phase of *Uca* chromatophores may be prolonged without any apparent effect upon the rhythmical cen-

ter itself. It is possible that activities other than those of the chromatophores might also be responsive to external factors while at the same time a certain minimal level is maintained by virtue of a basic rhythmicity. Such activities might be related to those cyclic events showing a longer period, such as reproduction and molting.

## SUMMARY

1. It has been shown that the frequency of the diurnal rhythm of *Uca* chromatophores is not altered to correspond to alternating light and dark periods of 16 hours each. This is true even though the animals are exposed to nine complete cycles.

2. When animals showing a normal chromatophoric rhythm which would persist in constant darkness are exposed to illumination beginning at 1:00 A.M. and ending either 6 or 12 hours later, a shift in the chromatophoric rhythm results. The shift is such that the most concentrated condition of the chromatophores occurs about 6 hours earlier than normal, and it persists as long as the animals are maintained in constant darkness after the single period of illumination.

3. When animals showing a normal chromatophoric rhythm which would persist in constant darkness are exposed to illumination beginning at any time in the day phase and ending at 7:00 A.M., a shift in the chromatophoric rhythm results. The shift is such that the most concentrated condition of the chromatophores occurs about 6 hours later than normal, and it persists as long as the animals are maintained in constant darkness after the single period of illumination. Under these conditions periods of illumination lasting 16, 20, or 24 hours are equally effective.

4. The persistence of both of the shifts of chromatophoric rhythm described has been interpreted as indicating corresponding shifts in the rhythm of the basic center.

5. Repeated exposures to illumination by night and to darkness by day are necessary to obtain reversal of the chromatophoric rhythm.

6. A single exposure to illumination at night of animals otherwise maintained in constant darkness effects, at most, a shift of approximately 6 hours in the rhythm. Such a shift then persists in constant darkness.

7. On the basis of the results reported here the basic rhythmicity has been characterized in terms of variations in sensitivity to light changes. These variations in sensitivity are discussed in relation to light changes to which the animals are normally exposed.

## LITERATURE CITED

BELING, I. 1929. Über das Zeitgedächtnis der Bienen. Zeitschr. f. vergl. Physiol., 9:239–337.

BENNITT, R. 1932. Diurnal rhythm in the proximal pigment cells of the crayfish retina. Physiol. Zoöl., 5:65–69.

BENTLEY, E. N.; GUNN, D. L.; and EWER, D. W. 1941. The biology and behavior of *Ptinus tectus*, a pest of stored products. I. The daily rhythm of locomotor activity. Jour. Exper. Biol., 18:182–95.

BREMER, H. 1926. Über die tageszeitliche Konstanz im Schlupftermine der Imagines einiger Insekten und ihre experimentelle Beeinflussbarkeit. Zeitschr. f. wiss. Insektenbiol., 21:209–16.

BROWN, F. A., JR., and WEBB, H. M. 1948. Temperature relations of an endogenous daily rhythmicity in the fiddler crab, *Uca*. Physiol. Zoöl., 21:371–81.

———. 1949. Studies of the daily rhythmicity of the fiddler crab, *Uca*. Modifications by light. Physiol. Zoöl., 22:136–48.

BÜNNING, E. 1932. Die Erblichkeit der Tagesperiodizität bei Pflanzen. Naturwiss., 20:340–45.

———. 1935. Zur Kenntnis der endonomen Tagesrhythmik bei Insekten und bei Pflanzen. Ber. d. deutsch. bot. Gesellsch., 53:394–623.

———. 1936. Die Entstehung der Variationsbewegungen bei Pflanzen. Ergebn. d. Biol., 13:235–347.

HOGBEN, L. T., and SLOME, D. 1931. The pigmentary effector system. VI. The dual character of endocrine coordination in amphibian colour change. Proc. Roy. Soc. London, B, 108: 10–53.

JAHN, T. L., and CRESCITELLI, F. 1940. Diurnal changes in the electrical response of the compound eye. Biol. Bull., 78:42–52.

JAHN, T. L., and WULFF, V. J. 1941*a*. Retinal pigment distribution in relation to a diurnal rhythm in the compound eye of *Dytiscus*. Proc. Soc. Exper. Biol. and Med., 48:656–60.

———. 1941*b*. Influence of a visual diurnal rhythm on flicker response contours of *Dytiscus*. *Ibid.*, 48:660–65.

———. 1943. Electrical aspects of a diurnal rhythm in the eye of *Dytiscus fasciventris*. Physiol. Zoöl., 16:101–9.

JOHNSON, M. S., 1926. Activity and distribution of certain wild mice in relation to biotic communities. Jour. Mammal., 7:245–77.

KALMUS, H. 1934. Über die Natur des Zeitgedächtnisses der Bienen. Zeitschr. f. vergl. Physiol. 20:405–19.

———. 1938. Das Aktogramm des Flusskrebs und sein Beeinflussung durch Organextrakte. *Ibid.*, 25:798–802.

KLEINHOLZ, L. H. 1937*a*. Studies in the pigmentary system of Crustacea. I. Color changes and diurnal rhythm in *Ligia baudiniana*. Biol. Bull., 72:24–36.

———. 1937*b*. Studies in the pigmentary system of Crustacea. II. Diurnal movements of the retinal pigments of Bermudan decapods. *Ibid.*, 73:176–89.

PARK, O., and KELLER, J. G. 1932. Preliminary analysis of activity rhythm in nocturnal forest insects. Ecology, 13:335–46.

ROBERTS, T. W. 1936. Preliminary analysis of activity in the crayfish, *Cambarus virilis*. Bull. Ecol. Soc. Amer., 17:27.

SCHALLEK, W. 1942. Some mechanisms controlling locomotor activity in the crayfish. Jour. Exper. Zoöl., 91:155–66.

SCHMITZ, N. 1934. Die periodische Bewegungen der Blätter von *Coleus penzigii*. Zeitschr. f. Bot., 27:355–411.

SMITH, R. I. 1948. The role of the sinus glands in retinal pigment migration in grapsoid crabs. Biol. Bull., 95:169–85.

STIER, T. J. B. 1933. Diurnal changes in activities and geotropism in *Thyone briareus*. Biol. Bull., 64:326–32.

WELSH, J. H. 1930. Diurnal rhythm of the distal pigment cells in the eyes of certain crustaceans. Proc. Nat. Acad. Sci., 16:386–95.

———. 1935. Further evidence of a diurnal rhythm in the movement of pigment cells in eyes of crustaceans. Biol. Bull., 68:247–52.

———. 1936. Diurnal movements of the eye pigments of *Anchistioides*. *Ibid.*, 70:217–27.

———. 1941. The sinus glands and twenty-four hour cycles of retinal pigment migration in crayfish. Jour. Exper. Zoöl., 86:35–49.

WULFF, V. J., and JAHN, T. L. 1943. Intensity-EMF relationships of the electroretinogram of beetles possessing a visual diurnal rhythm. Jour. Cell. and Comp. Physiol., 22:89–94.

YARWOOD, C. E., and COHEN, MORRIS. 1949. Diurnal responsiveness of *Erysipha graminis* to nutrients. Science, 110:477–78.

# CHROMATOPHOROTROPINS IN THE CENTRAL NERVOUS SYSTEM OF UCA PUGILATOR, WITH SPECIAL REFERENCE TO THEIR ORIGINS AND ACTIONS[1]

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THE central nervous system was first demonstrated to be a source of a chromatophorotropically active substance in crustaceans by Brown (1933). By the use of the shrimp *Palaemonetes*, it was shown that the injection of sea-water extracts of the central nervous system resulted in the concentration of red and yellow pigments of this species. It was concluded, further, that, although the central nervous system was a secondary source of this substance (the sinus gland being the primary source), some site in the anterior region of the cephalothorax was the primary source of

[1] This investigation is a portion of a thesis submitted in partial fulfilment of the requirements for the Ph.D. degree in biology at Northwestern University, in June, 1950. The author wishes to express her sincere appreciation to Dr. Frank A. Brown, Jr., for his guidance and helpful criticisms during the course of this investigation.

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a white-concentrating principle (Brown, 1935). By the use of the shrimp *Leander adspersus*, it was demonstrated that similar extracts of the central nervous system produced white-pigment concentration with an effectiveness less than that of extracts of the eyestalks (Knowles, 1939).

Comparisons of the responses of *Palaemonetes* with those of *Crago* to eyestalk removal and electrical stimulation led to a suggestion that, unlike *Palaemonetes*, *Crago* contained an extra-eyestalk source of a substance which acted antagonistically to a substance from the eyestalk (Brown, 1939). The existence of such a substance emanating from the nervous system which acted antagonistically to a substance from the sinus gland was first demonstrated in the crayfish, *Cambarus* (Brown and Meglitsch, 1940). Extracts of the sinus glands produced a dispersion of white pigment of this animal, while extracts of the central nervous system produced a concentration. When the eyestalks of *Crago* were removed and the stubs cauterized, the telson and uropods underwent a transitory darkening and then retained a lightened condition indefinitely. A similar tail-darkening could be produced by the injection of extracts of the connectives and commissures (Brown and Ederstrom, 1940). If animals whose tails had been darkened in this manner received an injection of eyestalk extract, the tails returned to the light condition in 3 or 4 minutes, while those of the uninjected controls remained dark.

Evidence that the central nervous system of *Crago* contained two chromatophorotropins was advanced by Brown and Wulff (1941). It was shown that strong electrical stimulation of the eyestubs of *Crago* resulted predominantly in body-lightening with tail-darkening, while a weaker stimulation resulted in simultaneous body-darkening and tail-darkening. An alcohol-soluble fraction of the connectives produced blanching of the trunk with no tail-darkening, while an alcohol-insoluble fraction produced less pronounced blanching of the trunk and simultaneous darkening of the telson and uropods. On the basis of these facts these authors postulated that one substance secreted by the central nervous system lightens all the body except the telson and the uropods and that a second substance darkens the telson and uropods and, in the absence of the first, darkens the rest of the body. Further support of this hypothesis was offered by the demonstration that the extent of body-lightening produced by extracts of connectives was inversely correlated with the extent of body-darkening which followed (Brown, 1946). By means of careful anatomical fractionation, the tritocerebral commissure was shown to be the source of the *Crago*-darkening hormone (CDH), while the *Crago*-body-lightening hormone (CBLH) appeared to be distributed throughout the central nervous system (Brown, 1946). In the following year a chemical separation of the two mutually antagonistic substances, CDH and CBLH, was effected (Brown and Klotz, 1947).

Both these substances were shown to be present in the central nervous system of *Palaemonetes*, but only one, CBLH, was demonstrated to occur in the brachyurans tested (Brown and Ederstrom, 1940). An extensive study of the comparative distribution in crustaceans confirmed the absence of CDH from brachyurans and described in some detail the relative distribution of the two substances within the nervous systems of other crustaceans studied (Brown and Saigh, 1946).

It appeared, therefore, that the Brachyura differed markedly from the other

crustaceans investigated, in that they contained within their nervous systems only one of these two chromatophorotropins. Few investigations of the influence of central nervous system extracts upon the chromatophore system of crabs have been made. However, it has been observed that extracts of the central nervous system of *Uca* were very effective in darkening eyestalkless animals of this species (Brown, 1948), and recently it has been shown that central nervous system extracts of another crab, *Hemigrapsus oregonensis*, caused dispersion of the melanin of animals of the same species (Bowman, 1949).

*Uca pugilator*, the fiddler crab, was chosen as the subject of the present investigation. Like the other brachyurans studied by Brown and Saigh (1946), it has been shown to contain one chromatophorotropin, the *Crago*-body-lightening principle, distributed throughout its central nervous system. Unlike many of the other crabs, *Uca* is capable of rather pronounced color changes. In addition to having a diurnal rhythm such that it is light at night and dark by day (Abramowitz, 1937; Brown and Webb, 1948), it has been shown to be capable of responses to light intensity, background, and temperature (Brown and Sandeen, 1948). Removal of the eyestalks from these animals causes the black pigment of the legs to become concentrated and the white pigment to become dispersed. These conditions are maintained indefinitely under constant conditions. It has been shown that animals in this state darken as a result of injections of extracts of the central nervous system (Brown, 1948). It is the purpose of this investigation to identify the number of active substances secreted by the central nervous system which are responsible for this action and to describe their distribution in the central nervous system and their characteristics of action.

## MATERIALS AND METHODS

The following investigation was conducted at the Marine Biological Laboratory, Woods Hole, Massachusetts, during the months of June, July, and August. The animals used for these experiments were specimens of *U. pugilator* collected in the vicinity of Woods Hole. The animals were maintained in the laboratory in a small amount of sea water in large white-enameled containers or large glass battery jars. The population consisted of individuals quite uniform in size, the average weight of the females being approximately 2.2 gm. and the average weight of the males about 3.2 gm.

## PREPARATION OF EXTRACTS

Normal animals were taken at random from the stock supply of *Uca*. A large portion of the dorsal region of the carapace was rapidly removed from an individual, and the central nervous system was exposed by clearing away the overlying viscera. With the aid of a dissecting microscope the circumesophageal connectives were cut near the point of their junction with the thoracic nerve cord, and the optic nerves were severed near their junction with the supra-esophageal ganglia. Then the supra-esophageal ganglia were grasped with jewelers' forceps and were carefully lifted out, carrying with them the connectives and the tritocerebral commissure, which joins together the two connectives about one-third the distance from the supra-esophageal ganglia to the thoracic nerve cord.

After the large nerves radiating from the thoracic nerve cord had been carefully cut as close as possible to the cord, it was possible to lift the cord out in one

piece and place it in a container of sea water. After ten nervous systems had been removed in this manner, the supra-esophageal ganglia were cut free from the connectives. The parts of the central nervous system thus obtained were placed in three clean and dry embryological dishes with ground-glass bottoms, one for the supra-esophageal ganglia, one for the connectives, and the last for the thoracic nerve cords. The total procedure could be completed in approximately 10 or 15 minutes.

After all excess sea water had been removed, the tissues were triturated thoroughly with a glass pestle. The ground tissue was mixed with 0.5 cc. of sea water. This constituted the stock extract, with a concentration of 1 portion of a central nervous system per 0.05 cc. Thus, when an animal was injected with the customary dose of 0.05 cc. of this concentration, it received an extract of one portion of a central nervous system, or, in other words, the equivalent of that part normally existing in one animal. Such a concentration will be referred to in the following account as a "concentration of 1."

Extracts of the sinus glands and of the eyestalks minus sinus glands were prepared in a similar manner. One procedure consisted of the removal of the sinus glands from the eyestalks of intact animals. In this operation a hole was made in the eyestalk just above the sinus gland, by carefully shaving away the exoskeleton with a sharp Bard-Parker scalpel. Under a dissecting microscope the sinus gland was readily visible in this opening as a small bluish-white body. By gentle pressure on the body, this gland could be forced out through the hole and then removed and placed in a ground-glass-bottomed embryological dish. After the two sinus glands of an individual had been removed, the eyestalks were cut off at their bases and placed in a second dish.

Five animals were operated on in this manner, and the resulting groups of five pairs of sinus glands and five pairs of eyestalks were each triturated and extracted in 0.75 cc. of sea water. This yielded concentrations of one pair of sinus glands and one pair of eyestalks minus sinus glands, respectively, per 0.15 cc. of sea water, which is a concentration of 1/3, since the usual dose would contain the material of one-third the complement of that tissue within a single organism.

An alternative method of preparation of extracts of sinus glands and of residual eyestalk tissue involved, first, severing the eyestalks from the animals and placing them in sea water in Syracuse dishes. The retina was first removed with a sharp scalpel, and then the exoskeleton was split longitudinally. The optic ganglia and optic nerve tract were lifted away from the exoskeleton in one piece and were suspended in sea water. The sinus gland was removed from its position among the optic ganglia and placed in one embryological watch glass, and the residual nervous tissue was placed in another. This method differed from the first, in that here only the nervous tissue of the eyestalks was compared with the sinus gland. As in the earlier experiments, the five pairs of sinus glands and optic ganglia were each triturated and extracted in 0.75 cc. of sea water to provide concentrations of 1/3, as described in the preceding paragraph.

### ASSAY OF EXTRACTS

For assay of the extracts it was essential to have, as assay animals, individuals whose chromatophore conditions were as uniformly responsive as possible. This was accomplished by removing the eyestalks from *Uca* 24 hours prior to the

time of their use as assay animals. The black pigment of these animals is usually completely concentrated, and the white pigment is completely dispersed. It was necessary to discard an occasional animal which deviated from this state. Groups of five assay animals were placed in finger bowls under controlled conditions of light intensity and temperature. The temperature was 26° ± 1° C., and the light intensity was approximately 50 foot-candles.

Each extract to be assayed was injected into the five animals in one finger bowl. Each individual was held with its ventral surface up, and the needle of the syringe was inserted at the base of the fourth or fifth walking leg in such a manner that the fluid was injected directly into the ventral hemocoele. The amount of extract injected into each animal was always 0.05 cc. Following the injection, the average state of dispersion of the black and white pigments of the chromatophores of the five animals was recorded at the end of 15 minutes and 30 minutes and at 30-minute intervals thereafter for the duration of the effect. The condition of the chromatophores was recorded by using a standard chromatophore index. According to this index, 1 signified maximum concentration of pigment, and 5 signified maximum dispersion, with 2, 3, and 4 indicating three intervening conditions. The chromatophores on the anterior surfaces of the legs were those used for determining the chromatophore state. The average chromatophore condition was rapidly estimated with a dissecting microscope.

### CALCULATION OF ACTIVITY

The activity of each extract was determined in the following manner: Since the value of the initial condition of the black chromatophores in these eyestalkless assay animals is expressed as 1, the amount of dispersion for any reading resulting from an influence of the extract is the average observed value *minus* 1. The sum of these corrected values for all readings in which a response was noted constitutes a measure of the activity of the extract. Each experiment was continued until the pigment had returned to the original condition. Therefore, the activity of an extract is seen to be a value which includes a measure of both the intensity and the duration of the response.

In the case of the white pigment the initial condition is one of complete dispersion, which is designated by the number 5. Therefore, the response resulting from the extract after any given interval would be 5 *minus* the average observed value. The sum of these corrected values for the duration of the experiment constitutes a measure of the activity of the extract on the white chromatophores.

### RELATIVE DISTRIBUTION AND CHARACTERISTICS OF DILUTION

A dilution series for each of the major portions of the central nervous system was prepared from the stock extract with a concentration of 1. (This concentration was defined in the section on materials and methods.) The concentrations obtained, in addition to the initial one, were 1/3, 1/9, 1/27, 1/81, and 1/243. An animal injected with a dose of 0.05 cc. of these concentrations would, therefore, receive 1/3, 1/9, 1/27, 1/81, or 1/243 the amount of that portion of the central nervous system normally existing in a single animal.

Similar dilution series of sinus glands and eyestalks minus sinus glands were made to give concentrations of 1/3, 1/9, 1/27, 1/81, 1/243, and 1/729. In three experiments the complete eyestalks except for sinus glands were compared with the sinus glands alone, whereas in a fourth experiment the nervous elements

of the eyestalk were extracted by themselves.

In order to assay any series of dilutions of a single organ, six dishes containing five assay animals each were necessary. The difficulties involved in reading such a number of animals in the specified time intervals made it necessary to limit any one experiment to the comparison of two organs. Consequently, experiments were repeated with various combinations of two of the organs to be studied until a total of four experiments for each had been completed.

With the use of the activity values obtained in these four experiments, an average activity value at each dilution for each organ was obtained. In the fourth experiment, in which only the optic ganglia were compared with sinus gland, activity values for optic ganglia were obtained which did not differ significantly from those values obtained for the whole eyestalk minus the sinus glands (the first three experiments). Therefore, it was concluded that the optic ganglia contain all the chromatophoric activity of the eyestalk outside of the sinus gland, and the activity values for the optic ganglia were obtained from the average of these four experiments.

The average activity values of the dilution series were used to construct the curves in Figures 1–5. It can be seen that all organs tested contain an active principle, which results in the dispersion of the black pigment, but that only one, the connectives, shows appreciable activity in the concentration of the white pigment. By comparing the activity values given by extracts of 1/3 concentration, the organs can be listed in decreasing order of effectiveness in causing black-pigment dispersion as follows: optic ganglia (25.5), sinus gland (22.8), supra-esophageal ganglia (20.1), thoracic nerve cord (16.8), connectives (6.4). The wet weights of these tissues were, respectively, 1.13, no value, 0.7, 3.2, and 0.39 mg. The relationship between these activity values and the relative concentra-

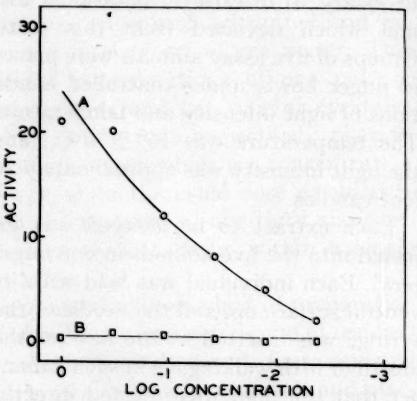


FIG. 1.—The relationships between the average activity values for, *A*, black chromatophores and, *B*, white chromatophores of *Uca* and the logarithm of concentration for extracts of supra-esophageal ganglia.

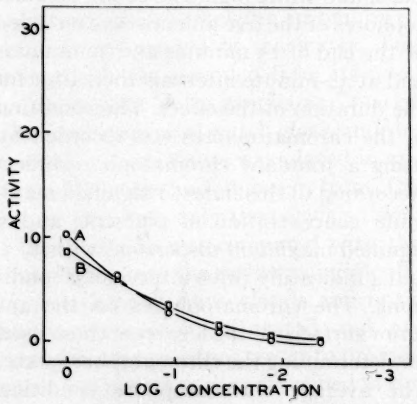


FIG. 2.—The relationships between the average activity values for, *A*, black chromatophores and, *B*, white chromatophores of *Uca* and the logarithm of concentration for extracts of connectives.

tions in the various portions of the nervous system, if a single active substance is involved in black-pigment dispersion, may be readily calculated from the dilution curves and the wet weights of the

portions. Such calculations lead to the indication of almost a hundred fold difference in the apparent concentration between the most and the least active regions. It was not deemed worth while,

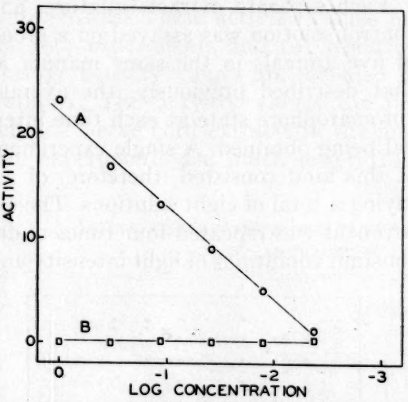


FIG. 3.—The relationships between the average activity values for, *A*, black chromatophores and, *B*, white chromatophores of *Uca* and the logarithm of concentration for extracts of thoracic nerve cord.

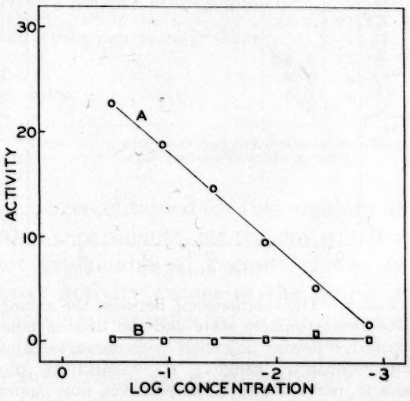


FIG. 4.—The relationships between the average activity values for, *A*, black chromatophores and, *B*, white chromatophores of *Uca* and the logarithm of concentration for extracts of sinus gland.

however, to include such calculated values here, inasmuch as (as will be demonstrated later) the activity observed is actually a resultant of the activities of two mutually antagonistic principles.

Concentrations of the organs other than the connectives which show a black-dispersing activity of 10 or less, comparable to the black-dispersing activity exhibited by the connectives, are seen not to exhibit significant white-concentrating activity, in contrast to the connectives. It appears, therefore, that these two activities are produced by two separate substances. Furthermore, the fact

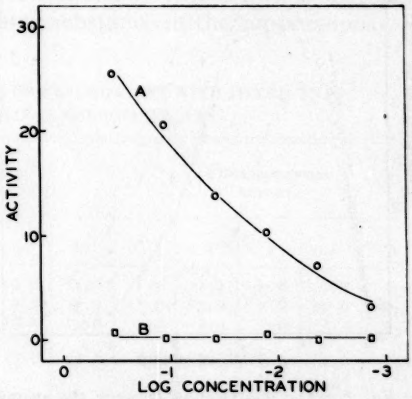


FIG. 5.—The relationships between the average activity values for, *A*, black chromatophores and, *B*, white chromatophores of *Uca* and the logarithm of concentration for extracts of optic ganglia.

that there is no relationship between black-dispersing and white-concentrating activity common to the dilution curves of all the organs tested lends support to this hypothesis.

## CHROMATOPHORE ACTIVITY OF COMBINATIONS OF PARTS OF THE CENTRAL NERVOUS SYSTEM

Animals were taken at random from the stock supply of *Uca* maintained in the white-enameled containers. The central nervous systems were dissected as described previously, and stock extracts of the supra-esophageal ganglia, connectives, and thoracic nerve cord with concentrations of 1 were prepared (twelve organs per 0.6 cc. or one organ per 0.05

cc. sea water). Then 0.2 cc. of each of the three stock extracts was diluted with 0.2 cc. of sea water, giving a concentration of 1/2. Thereafter, three mixed extracts were prepared in such a manner that one

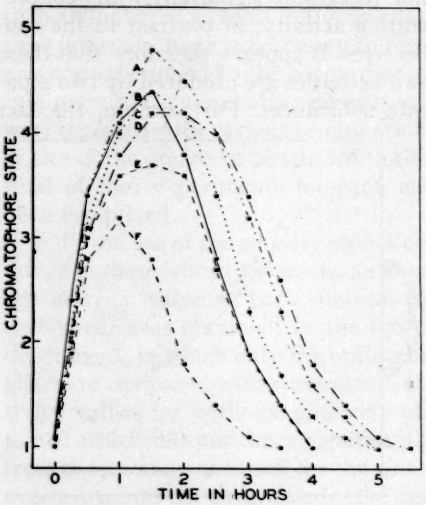


FIG. 6.—The relationships between the average black chromatophore state and the time in hours obtained following injection with extracts of *A*, supra-esophageal ganglia; *B*, connectives plus thoracic nerve cord; *C*, connectives plus supra-esophageal ganglia; *D*, supra-esophageal ganglia plus thoracic nerve cord; *E*, thoracic nerve cord; *F*, connectives.

contained 0.2 cc. of the stock supra-esophageal ganglia extract plus 0.2 cc. of the stock connectives extract; the second contained 0.2 cc. of the stock supra-esophageal ganglia extract plus 0.2 cc. of the stock thoracic nerve cord extract; and the third contained 0.2 cc. of the stock thoracic nerve cord extract plus 0.2 cc. of the stock connectives extract. All possible combinations of two were, therefore, obtained, and each mixed extract contained a concentration of 1/2 dilution of each organ, making it possible to compare them with the extracts of 1/2 dilution in sea water of each organ. Two controls were used in this experiment. One consisted of a volume of muscle tissue approximately equal to that of the thoracic nerve cord tissue and extracted in sea water. The second control was sea water alone.

Each separate extract, mixture, and control solution was assayed on a group of five animals in the same manner as that described previously, the average chromatophore state at each time interval being obtained. A single experiment of this kind consisted, therefore, of assaying a total of eight solutions. The experiment was repeated four times under constant conditions of light intensity and

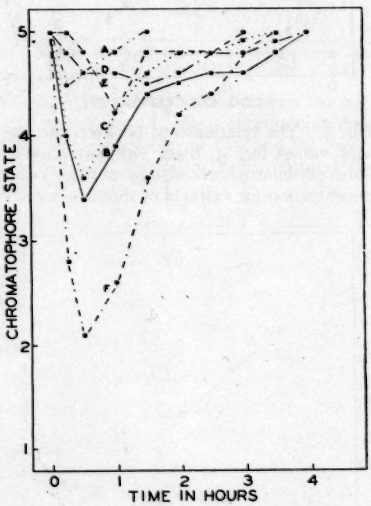


FIG. 7.—The relationships between the average white chromatophore state and the time in hours obtained following injection with extracts of *A*, supra-esophageal ganglia; *B*, connectives plus thoracic nerve cord; *C*, connectives plus supra-esophageal ganglia; *D*, supra-esophageal ganglia plus thoracic nerve cord; *E*, thoracic nerve cord; *F*, connectives.

temperature, similar to those of the previous experiments.

From these four experiments the average time course of response was determined, and these values were used to plot the curves in Figures 6 and 7.

Neither of the controls is included in these figures, since they gave no appreciable chromatophoric response.

The black-dispersing and white-concentrating activity values were calculated for each of the extracts and the two controls. These activity values for the four experiments and the resulting average activity values are included in Table 1. The activity values for the thoracic nerve cord extracts in the first two experiments are much lower than (16.2), thoracic nerve cord (13.8), and connectives (7.9). A similar listing can be made in order of decreasing power to cause concentration of white pigment: connectives (10.7), connectives plus thoracic nerve cord (5.9), supra-esophageal ganglia plus connectives (4.1), supra-esophageal ganglia plus thoracic nerve cord (2.4), thoracic nerve cord (1.7), and supra-esophageal ganglia (1.2).

These results demonstrate clearly that some substance in the supra-esophageal

TABLE 1

ACTIVITY VALUES OBTAINED IN THE SERIES OF EXPERIMENTS WITH MIXED AND SIMPLE EXTRACTS OF THE CENTRAL NERVOUS ORGANS

| Tissue | Black-dispersing Activity | | | | | White-dispersing Activity | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | Av. | 1 | 2 | 3 | 4 | Av. |
| Supra-esophageal ganglia | 25.5 | 21.5 | 18.4 | 19.8 | 21.3 | 1.2 | 1.4 | 0.8 | 1.4 | 1.2 |
| Connectives | 9.9 | 9.7 | 4.8 | 7.2 | 7.9 | 11.0 | 13.2 | 7.6 | 10.6 | 10.7 |
| Thoracic nerve cord | *9.5 | *5.8 | 19.2 | 20.8 | 13.8 | 3.2 | 2.4 | 0.0 | 1.2 | 1.7 |
| Supra-esophageal ganglia plus connectives | 22.3 | 14.1 | 16.8 | 17.2 | 17.6 | 2.6 | 5.8 | 3.4 | 4.6 | 4.1 |
| Supra-esophageal ganglia plus thoracic nerve cord | 22.1 | 21.4 | 17.4 | 22.2 | 20.8 | 4.8 | 3.8 | 0.4 | 0.8 | 2.4 |
| Connectives plus thoracic nerve cord | 13.4 | 14.5 | 16.8 | 20.0 | 16.2 | 6.8 | 11.2 | 3.4 | 2.2 | 5.9 |
| Muscle | 1.0 | 0.0 | 0.0 | 0.0 | 0.2 | 1.8 | 0.6 | 1.0 | 0.0 | 0.8 |
| Sea water | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.8 | 0.0 | 2.0 | 0.0 | 0.7 |

* These values were lower than any that have been obtained in numerous other experiments with thoracic nerve cord extracts for reasons which are not explainable at the present.

have been obtained for this organ in any other experiments, for reasons which are not explainable at present. These two lower activity values of the simple extracts are not reflected in the mixed extracts containing thoracic nerve cord, for reasons which are equally obscure.

Using the average activity values in Table 1, it is possible to list the simple extracts and the mixed extracts in the following order of decreasing power to cause dispersion of black pigment: supra-esophageal ganglia (21.3), brain plus thoracic nerve cord (20.8), supra-esophageal ganglia plus connectives (17.6), connectives plus thoracic nerve cord

ganglia and thoracic nerve cord influences the expression of the white-concentrating substance, and they also suggest that some substance in the connectives decreases the black-dispersing action of the other two portions.

The black activity of the supra-esophageal ganglia plus thoracic nerve cord extract does not differ appreciably from the activity obtained with the simple extract of the supra-esophageal ganglia (20.8 and 21.3, respectively). Although activity values of 25 have been obtained with some extracts in this study, this value may well be in the range of the limitations of the method of assay used.

In a physiological assay of this sort the animals appear to be capable of increased response with increasing concentration of active material up to a certain point; any concentrations above this elicit no greater response. Therefore, although a mixed extract of thoracic nerve cord and

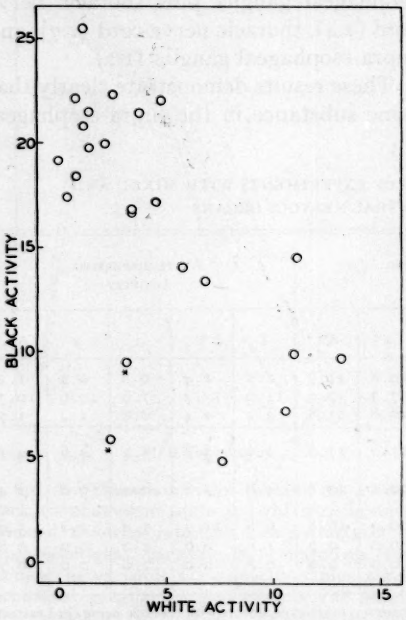


FIG. 8.—A scatter plot in which the average values for black-dispersing activity of all the simple and mixed extracts of central nervous organs are plotted against the average values for white-concentrating activity obtained with the same extracts. *Thoracic nerve cord extracts which give unusually low black-dispersing activity values.

supra-esophageal ganglia contains a greater amount of active material than does a simple extract of either organ separately, it may well be impossible to demonstrate the greater quantity as a result of the physiological limits of response of the animals.

In an effort to demonstrate whether these results are explainable in terms of a hypothesis that the black-dispersing and white-concentrating principles are mutually antagonistic, Figure 8 was prepared. In this figure white-concentrating activity is plotted against black-dispersing activity for all the experiments which used simple and mixed extracts of the central nervous system. The relationship obtained indicates that the greater the amount of black-dispersing activity given by any extract, the less the amount of white-concentrating activity given by that extract, and vice versa.

To clarify further the character of the interrelationship existing between the black-dispersing principle and the white-concentrating principle, a brief experiment was designed in which a series of extracts was made containing the same concentration of the white-concentrating principle from the connectives but varying concentrations of the black-dispersing principle from the "brain." This was achieved by first making a connective and commissure extract with a concentration of 1. To do this, fourteen pairs of connectives with their commissures were extracted in 0.7 cc. of sea water. Then a supra-esophageal ganglia extract with a concentration of 1 (fourteen supra-esophageal ganglia in 0.7 cc. of sea water) was made, and, after retaining 0.2 cc. of this, subsequent dilutions were made which yielded concentrations of 1/3, 1/9, 1/27, 1/81, and 1/243. Mixtures were then obtained by using 0.2 cc. of the various supra-esophageal ganglia concentrations and 0.1 cc. of the stock extract of connectives. The resulting concentrations were as follows: in Extract I, 2/3 supra-esophageal ganglia plus 1/3 connectives; in Extract II, 2/9 supra-esophageal ganglia plus 1/3 connectives; in Extract III, 2/27 supra-esophageal ganglia plus 1/3 connectives; in Extract IV, 2/81 supra-esophageal ganglia plus 1/3 connectives; in Extract V, 2/243 supra-esophageal ganglia plus 1/3 connectives; in Extract VI, 2/729 supra-esophageal

ganglia plus 1/3 connectives. Each of these extracts was assayed on a group of three assay animals, and the activity values were calculated. These activity values of the series of extracts were used to construct the curves in Figure 9.

The antagonistic action of the black-dispersing substance on the expression of the white-concentrating substance is readily visible in Figure 9, where it can be seen that the higher the concentration of black-dispersing substance, the lower is the amount of white concentration obtained. This relationship appears to be linear, although the fact that only three animals were used in the assays prevents any definite conclusion in this respect. However, in the previous dilution experiments it was found that the 1/243 concentration of supra-esophageal ganglia gave very little black-dispersing activity (a value of 2.1); and consequently it appears that the activity values for both black-pigment dispersion and white-pigment concentration for the more dilute extracts of supra-esophageal ganglia in this experiment must become increasingly the result of the contribution of active substances by the connectives.

In the previous dilution experiments it was found that the average activity values at a concentration of 1/3 for white-pigment concentration ranged from 4.2 to 7.7, with an average value of 6.0. The use of any one of these values in the curve obtained in this experiment could not be justified because of the difference in the number of animals involved. However, they do serve as an indication of the white-pigment-dispersing activity that might have been obtained in the absence of the added amounts of black-dispersing substance. It is to be noted that all these values are greater than those which were obtained in this experiment.

With regard to black-pigment dispersion, nothing definitive can be concluded as to the shape of the curve, although it seems probable that it is not a straight line. The black-pigment-dispersing activity values for 1/3 dilution extract of connectives in the previous dilution experiments ranged from 5.0 to 7.6, with an average value of 6.4. This gives some indication of where a point on the curve might occur if a 1/3 concentration of this extract of connectives had been assayed. No specific support for an antagonistic influence of the white-concentrating substance on the expression of the black-dispersing substance can be drawn from this brief experiment.

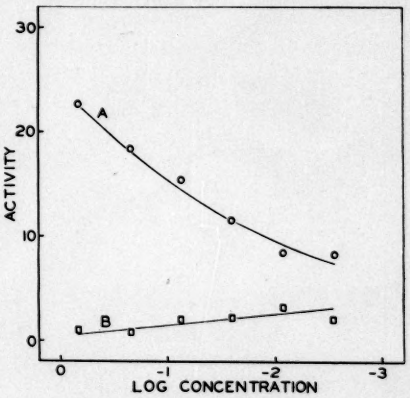


FIG. 9.—The activities for *A*, black chromatophores and *B*, white chromatophores are plotted against the logarithm of concentration of supra-esophageal ganglia extract in mixtures containing a constant concentration of connective extract.

### DURATION OF POTENCY OF EXTRACTS

In many of the tissues whose extracts exhibited a white-concentrating activity there appeared to be much less consistency in magnitude of response from one experiment to another than for the black-dispersing activity. In order to determine to what extent this variation might be explained in terms of a rapid destruction of the white-concentrating substance

while *in vitro*, the following preliminary experiments were conducted.

Twenty pairs of connectives were dissected out rapidly and extracted in 1.00 cc. of sea water, giving a concentration of 1, as defined in "Materials and Methods"; 0.05 cc. of this extract was immediately injected into *Uca* whose eyestalks had been removed 24 hours earlier. The remaining extract was kept in the syringe at room temperature (23° C.) and again assayed at 15 minutes, 30 minutes, 60 minutes, 120 minutes, and 240 minutes. Each of these assays was made on three animals, and the average chromatophore condition of each group was determined at the same time intervals as described previously for the duration of the response. The black-dispersing and the white-concentrating activities of the extract were calculated for each time interval that a new assay was begun.

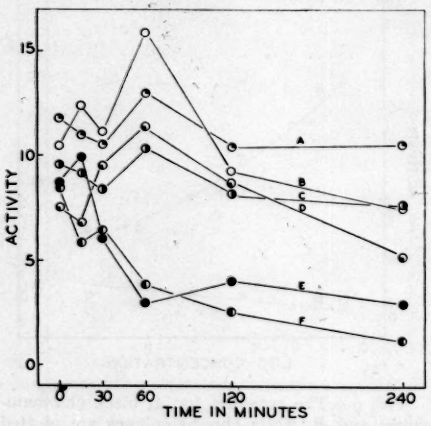


FIG. 10.—The relationship between the activity of extracts of connectives and the time in minutes of standing at room temperature. *A*, black-dispersing activity of boiled extract in experiment 1; *B*, black-dispersing activity of raw extract in experiment 2; *C*, black-dispersing activity of raw extract in experiment 1; *D*, white-concentrating activity of boiled extract in experiment 2; *E*, white-concentrating activity of raw extract in experiment 1; and *F*, white-concentrating activity of raw extract in experiment 2.

A second experiment was conducted in a similar manner. However, in this case the duration of potency of a raw extract was compared with that of a boiled extract. Twenty-two connectives were dissected rapidly and extracted in 3.3 cc. of sea water to give a concentration of 1/3. This extract was then divided in half; one half was assayed in the raw condition, and the other was first boiled for 1 minute. The extracts were assayed on five animals after the same time intervals as in the previous experiment. Both extracts were kept at a room temperature of 23° C. for the duration of the experiment.

The activity values for black-pigment dispersion and white-pigment concentration for each time interval that the extracts were assayed were used to construct the curves in Figure 10. From this figure it is evident that standing at room temperature as a sea-water extract results in a rapid decrease in potency of the white-concentrating substance. The rate is such that within an hour the activity has dropped to a value less than half the original. It is also apparent that the black-dispersing substance does not show a correspondingly rapid decrease in potency on standing. A dropoff in activity appears not to begin until after an hour has elapsed, and even at the end of 4 hours the activity is still only slightly less than the original value. It should be noted that in the first experiment an appreciable increase in black-dispersing activity was obtained at the end of an hour, and a similar tendency was indicated in the second experiment.

Boiling the extract in the second experiment caused not only a delay in the onset of the degeneration of potency of the white-concentrating substance to sometime beyond an hour but also a significant increase in activity during the first hour of standing. The black-dispers-

ing activity was slightly increased by boiling. The curves obtained for black-dispersing activity in the second experiment parallel one another, with all the points on the curve for the boiled extract being approximately two activity units higher than those for the raw extract.

These results indicate that an inactivation of the white-concentrating substance after extraction could play some part in the variation that was noticed from experiment to experiment. However, since the extracts were prepared rapidly and injected after approximately the same length of time had elapsed in each experiment conducted, the error as a result of such inactivation was probably small.

## DISCUSSION

The results of the experiments described in this report establish beyond a reasonable doubt the existence of two chromatophorotropins in the central nervous system of the fiddler crab. From the data obtained in the experiments on the dilution of the parts of the central nervous system, it was seen that the relationship between the log concentration and the black-dispersing activity could be represented by straight lines with similar slopes for the sinus gland and the thoracic nerve cord. This relationship for the optic ganglia and the supra-esophageal ganglia appeared not to be a simple straight line. In the case of the connectives the relationship between log concentration and black-dispersing activity, as well as white-concentrating activity, was even more obviously to be represented by curves. In addition to the latter fact, the connectives were seen to show the only appreciable white-concentrating activity. Since there is no evidence to the contrary, it seems reasonable to assume that the black-dispersing substance is the same in all the parts tested and, on the basis of this, that the behavior of their extracts on dilution is a function of some interrelationship between the two substances which have been demonstrated.

There is a strong suggestion from these results that a mutual antagonism exists between the black-dispersing and the white-concentrating substances. The relationship between the log concentration and the black-dispersing activity of the connectives deviates from a linear one, in all probability through the influence of the antagonistic action of the white-concentrating substance, which tends to prevent the complete expression of the actual amount of black-dispersing substance present. The white-concentrating substance appears to exist in the connectives in relatively high concentration in comparison with the black-dispersing substance.

In contrast to this situation, the definite straight-line character of the dilution curves in the cases of the thoracic nerve cord and the sinus gland suggests that a high concentration of black-dispersing substance exists and that there is a virtual absence of white-concentrating substance. Close examination of the dilution data for the optic ganglia and the supra-esophageal ganglia reveals that these curves tend to deviate from straight lines in a manner somewhat approaching that of the dilution curve of the connectives. That this tendency is not so striking as that shown in the connectives probably results from the fact that, unlike the connectives, there is a great predominance of the black-dispersing substance.

There is, however, some evidence that the deviation from a simple straight-line relationship in these cases could actually also be due to antagonistic action of white-concentrating substance present. Preliminary exploratory experiments

have shown that both the optic ganglia and the supra-esophageal ganglia possess substantial white-concentrating activity, which is normally masked in the total extracts. If, for example, the optic tract is separated from the optic ganglia and extracts of these two parts are assayed, the optic nerve tract shows significant white-concentrating activity, with very little black-dispersing activity, while the ganglia show only black-dispersing activity. In the experiments reported here no attempt was made to consider in any detail these two parts separately. The part referred to as the "optic ganglia" ordinarily also contained much of the optic nerve tract, which is present in the eyestalks of these animals.

The existence of white-concentrating activity in the supra-esophageal ganglia has similarly been demonstrated, in this case by a dissection procedure in which the supra-esophageal ganglia sheath is separated from the underlying nervous tissue. In experiments of this kind the supra-esophageal ganglia were placed in a small amount of sea water on a depression slide and the sheath was separated from the nervous tissue with the aid of forceps. The sheath, nervous tissue, and sea water were assayed, and in many cases both the sheath and the sea-water wash were shown to exhibit white-concentrating activity, while the nervous tissue showed only black-dispersing activity. Here, too, as in the eyestalks, the white-concentrating activity is apparently normally masked in the total extracts.

These experiments, which have shown that the white-concentrating substance does exist in the supra-esophageal ganglia and optic ganglia, are not reported more extensively here because they are still in the exploratory stages and have not been refined sufficiently to eliminate wide variations in the results obtained.

In the experiments using mixed extracts of the various parts of the central nervous system it was shown clearly that in the presence of a high concentration of black-dispersing substance the expression of the white-concentrating activity was depressed. The addition of extracts of supra-esophageal ganglia or thoracic nerve cord to extracts of connectives decreased the white-concentrating activity to a value approximately half that obtained with the pure extract of the connectives. This result was substantiated in the special experiment on antagonism, where it was shown that, as the concentration of the black-dispersing substance decreased, the white-concentrating activity increased.

Results from the experiments with mixed extracts suggested also that complete expression of the black-dispersing substance was prevented by a high concentration of the white-concentrating substance. The addition of extracts of connectives to extracts of supra-esophageal ganglia or thoracic nerve cord resulted in a black-dispersing activity value lower than that obtained with the simple extract of supra-esophageal ganglia. Furthermore, when the black-dispersing activity of the mixed and simple extracts was plotted against the white-dispersing activity, it was found that these activities were inversely related. In general, the greater the amount of black-dispersing activity obtained from any extract, the less the amount of white-concentrating activity obtained. These results support the hypothesis that these two chromatophorotropins of the central nervous system—the white-concentrating principle and the black-dispersing principle—are mutually antagonistic.

The independent nature of these two chromatophorotropins was demonstrated further in the experiment on the duration of potency *in vitro* in the form of extracts

of the connectives. The lack of reduction in black-dispersing activity and, in some extracts, the significant increase in activity which occurred during the first hour of standing in the unboiled extracts suggest that this principle is being produced from some inactive precursor. The increase in activity produced by boiling would suggest that the reduction in activity was retarded, while a complete activation of the precursor resulted from the treatment. Similarly, the inactivation of the white-concentrating substance apparently was retarded by boiling, to such an extent that the formation of the active form from an inactive precursor became evident only after this treatment.

In a study on the comparative distribution of *Crago*-darkening hormone and *Crago*-body-lightening hormone by Brown and Saigh (1946) it was shown that Brachyura contained only CBLH in their central nervous systems, while the other crustaceans studied contained both these principles. Whether one of the two substances which have been demonstrated in this study to exist in the central nervous system of *Uca* is identical to CBLH has not been ascertained at the present. However, it is clear that *Uca*, like the other crustaceans, contains more than one chromatophorotropin in its central nervous system, although CDH is definitely not present.

Since the chromatophores of the assay animals which were used in these experiments were in an initial condition in which the black pigment was completely concentrated and the white pigment was completely dispersed, it was impossible to demonstrate directly a black-pigment-concentrating action or a white-pigment-dispersing action of central nervous system extracts. However, exploratory experiments using normal animals whose white chromatophores were in intermediate conditions of dispersion have shown the existence of a white-dispersing action. The pigment of these chromatophores responded to injections of supraesophageal ganglia or thoracic nerve cord extracts by further dispersion. There is no evidence at present to indicate that this is not caused by the same principle which produces black-pigment dispersion. All efforts to demonstrate the existence of a black-pigment-concentrating principle have thus far failed.

Although it has been possible to demonstrate the existence of two chromatophorotropins in the central nervous system of *U. pugilator*, it is a rather difficult matter to show that these substances function in the normal color changes which characterize this animal. These normal responses include a diurnal rhythm of color change and a response to albedo, the ratio of incident light to reflected light, both of which may be under the control of the chromatophorotropins from the central nervous system, from the sinus gland, or from both sources. A method which may prove valuable in this respect is a demonstration of variations in concentrations of the active substances within the secretory tissues correlated with the chromatophore condition of the animals. It is possible that some of the variation in chromatophore activity from one experiment to another in this study may be explainable in terms of such functional changes. Although the animals used in these experiments as donors of nervous systems were all in the same phase of the diurnal rhythm, they were all subject to variations in light intensity. The crowded conditions in the containers in which the animals were kept were such that they were piled upon one another and were quite active most of the time. Not only the incident light but the background as well were, therefore, constantly varying.

SUMMARY

1. Two chromatophorotropins have been shown to exist in extracts of the central nervous system of *U. pugilator*. A principle which results in the dispersion of black pigment is present in high concentrations in the optic ganglia and supra-esophageal ganglia and in lower concentrations in the thoracic nerve cord and connectives. A second principle, which effects concentration of white pigment, is present in high concentration in the connectives, in significant but unmeasured concentrations in the optic ganglia and supra-esophageal ganglia, and is virtually absent from the thoracic nerve cord.

2. An antagonistic action was shown to exist between these two principles such that the presence of a large amount of black-dispersing principle decreased the expression of the white-concentrating principle. There was a suggestion that this antagonism was, furthermore, a mutual one, with the presence of a large amount of white-concentrating principle decreasing the expression of the black-dispersing principle.

3. The potency of the white-concentrating substance in raw extracts of the connectives was shown to decrease rapidly on standing at room temperature; the black-dispersing factor disappeared much more slowly. Brief boiling of the extracts retarded both. It was suggested that the maintenance and even a transitory increase in activity of the black-dispersing substance in both unboiled and boiled extracts of connectives and a transitory increase in activity of the white-concentrating substance in boiled extracts were the result of a formation of these substances from inactive precursors present in the extracts.

LITERATURE CITED

ABRAMOWITZ, A. A. 1937. The chromatophorotropic hormone of the Crustacea; standardization, properties and physiology of the eye-stalk glands. Biol. Bull., **72**:344–65.

BOWMAN, T. E. 1949. Chromatophorotropins in the central nervous organs of the crab, *Hemigrapsus oregonensis*. Biol. Bull., **96**:238–45.

BROWN, F. A., JR. 1933. The controlling mechanism of chromatophores in *Palaemonetes*. Proc. Nat. Acad. Sci., **19**:327–29.

———. 1935. Control of pigment migration within the chromatophores of *Palaemonetes vulgaris*. Jour. Exper. Zoöl., **71**:1–15.

———. 1939. Humoral control of crustacean chromatophores. Amer. Nat., **73**:247–55.

———. 1946. The source and activity of *Crago*-darkening hormone (CDH). Physiol. Zoöl., **19**: 215–23.

———. 1948. Hormones in crustaceans. In: The hormones: chemistry, physiology, and applications. Vol. 1, chap. v. New York: Academic Press.

BROWN, F. A., JR., and EDERSTROM, H. E. 1940. Dual control of certain black chromatophores of *Crago*. Jour. Exper. Zoöl., **85**:53–69.

BROWN, F. A., Jr., and KLOTZ, I. M. 1947. Separation of two mutually antagonistic chromatophorotropins from the tritocerebral commissure of *Crago*. Proc. Soc. Exper. Biol. and Med., **64**: 310–13.

BROWN, F. A., JR., and MEGLITSCH, A. 1940. Comparison of the chromotophorotropic activity of insect corpora cardiaca with that of crustacean sinus glands. Biol. Bull., **79**:409–18.

BROWN, F. A., JR., and SAIGH, L. M. 1946. The comparative distribution of two chromatophorotropic hormones (CDH and CBLH) in crustacean nervous systems. Biol. Bull., **91**:170–80.

BROWN, F. A., JR., and SANDEEN, M. I. 1948. Responses of the chromatophores of the fiddler crab, *Uca*, to light and temperature. Physiol. Zoöl., **21**:361–71.

BROWN, F. A., Jr., and WEBB, H. M. 1948. Temperature relations of an endogenous daily rhythmicity in the fiddler crab, *Uca*. Physiol. Zoöl., **21**:371–81.

BROWN, F. A., JR., and WULFF, V. J. 1941. Chromatophore types in *Crago* and their endocrine control. Jour. Cell. and Comp. Physiol., **18**:339–53.

# NUTRITION OF THE EUROPEAN CORN BORER, PYRAUSTA NUBILALIS (HBN.). II. SOME EFFECTS OF DIET ON LARVAL GROWTH CHARACTERISTICS[1]

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THE characteristics of arthropod growth have been in dispute since the latter part of the nineteenth century. Brooks (1886), Dyar (1890), and Przibram and Megusar (1912) postulated that arthropod growth is geometric in nature and presented simple constants to express changes in weight and linear dimensions associated with instar progression. There exists a large body of literature dealing with the application of the "Rules" of Dyar and Przibram to insect growth, and in a great many instances these rules have not seemed to apply satisfactorily. In the present paper no attempt will be made to review and discuss all the existing literature on the characteristics of insect growth. An attempt has been made, however, to include the principal publications applicable to the phases of the problem being investigated. The extensive work of Przibram, Bodenheimer, and others on the growth of paurametabolous forms has not been included, since Ludwig (1932 and 1934), Trager (1935), and Abercrombie (1936) have shown that the postulates of Przibram and Megusar (1912) and of Bodenheimer (1933) cannot be applied successfully to the growth characteristics of holometabolous insects. Most of the work on insect growth has been of an observational nature, and very little experimental work has been done on this problem.

During the past decade a fair knowledge has been attained concerning the nutritional requirements of several insect species. Little is known, however, concerning the effects of nutrition on growth and developmental patterns. Since growth, in terms of weight and instar progression, of European corn borer larvae can be varied by changes in the dietary conditions under which the larvae are reared, nutrition studies offer an experimental approach to the problem of growth characteristics.

## MATERIAL AND METHODS

In this study, larvae of the European corn borer were reared from egg to adult under aseptic culturing conditions. The basal dietary medium was only slightly modified from that reported in an earlier publication (Beck, Lilly, and Stauffer, 1949). Its composition is presented in Table 1.

The deficient diets used in these experiments represented the basal diet with various constituents omitted. The deficient diets were made approximately isocaloric with the basal diet by increasing the glucose content sufficiently to compensate for the omission of the other constituents. In the case of glucose-deficient diets, the glucose was replaced by cellulose, which has been shown to be indigestible (Beck, Lilly, and Stauffer, 1949).

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In the preparation of lipid-deficient diets, the various components were extracted with either diethyl ether or toluene for ten hours in a Soxhlet extractor.

The methods used in preparing the media and maintaining the cultures have been described in detail elsewhere (Beck and Stauffer, 1950) and need be only summarized here. Egg masses of the microörganisms, but these were discarded, and the data collected from them were disregarded. The final number of larvae in each culture was usually about seventeen. Rearing was carried out at 30° C. On the basal diet the larvae grew rapidly and matured in from 17 to 25 days. Normal pupation and adult emergence were observed.

TABLE 1

COMPOSITION OF A PURIFIED DIET ALLOWING SATISFACTORY GROWTH AND MATURATION OF EUROPEAN CORN BORER LARVAE

| Function | Substance | Amount (Gm.) | Per Cent of Dry Diet |
|---|---|---|---|
| Inert carrier | Distilled water | 125.0 (ml.) | 0.0 |
| | Bacto-Agar | 3.350 | 15.3 |
| | Cellulose (fibrous) | 2.700 | 12.3 |
| Carbohydrate | Glucose | 5.475 | 25.0 |
| Protein | Casein* | 5.475 | 25.0 |
| Lipids | Cholesterol | 0.219 | 1.0 |
| | Linoleic acid (containing 1 per cent A. tocopherol) | 0.219 | 1.0 |
| Minerals | Wesson's salts | 0.437 | 2.0 |
| Vitamins | Brewers'-yeast powder† | 2.188 | 10.0 |
| | Choline chloride | 0.087 | 0.4 |
| Unknown factor(s) | Leaf-factor preparation‡ | 1.750 | 8.0 |
| Total | | 21.900 | 100.0 |

* "Vitamin Test" casein, General Biochemicals, Inc.
† General Biochemicals, Inc.
‡ Supplied by Cerophyl Laboratories, Inc. This extract replaced the lyophilized corn leaves used in earlier work.

European corn borer were separated by incubating them in a dilute trypsin solution. The single eggs so obtained were rinsed thoroughly, were surface-sterilized in a solution containing 2 per cent sodium hydroxide and 2 per cent formaldehyde, and were introduced into sterile shell vials containing the dietary media.

Twenty-five eggs, each in a separate rearing vial, were started on each diet; on the average about twenty eggs hatched. In nearly every culture a few vials proved to be contaminated with

During the course of the experiments daily inspections were made in order to follow mortality, instar progression, and pupation. The experiments were usually terminated at the end of the twenty-first culture day. At this time the larvae and pupae were weighed and the pupae set aside for adult emergence. Except for the pupation molt, the larval head capsules were left intact when the insects underwent ecdysis. These head capsules were removed at the end of the experiment, and their maximum widths were meas-

ured to the closest one-hundredth of a millimeter. It was thus possible to obtain growth curves in the various experiments by plotting head-capsule widths against either instar number or time. In a few experiments the larval weight gain was followed by removing samples of larvae from the cultures and weighing them following ecdysis. All experiments were run at least twice, and many of them have been repeated several times.

## RESULTS

In the work by Beck, Lilly, and Stauffer (1949), involving the development of the basal diet, growth rate in terms of weight change was used as the primary criterion for determining dietary adequacy. In the present paper, weight changes will not be considered except in connection with the relationships between weight and linear dimensions. The growth characteristics to be considered at this time include the effect of diet on molting frequency (instar progression), head-capsule width, and head-width progression factors.

*Effect of diet on instar progression.*—The omission of different constituents of the basal diets reduced the frequency of molting to different degrees. Figure 1 shows the effects of several single constituent deficiencies on instar progression.

On the basal diet, the larvae molted frequently and attained the fifth (final) instar in from 10 to 14 days. In the absence of either yeast powder or lipids, molting was suppressed, and all larvae died before reaching the third instar. Diets containing no casein allowed poor growth, but many of the larvae attained the fifth instar by the twenty-first day. Diets in which the casein was omitted cannot be considered to have been protein-free, because of the protein contained in the brewers'-yeast powder. It is probable that on a protein-free diet, growth suppression would have approximated that observed in the yeast and lipid deficiencies. In the absence of glucose (replaced by cellulose) instar progression was retarded. When the source of leaf factor was omitted from the diet, instar progression was normal up to the

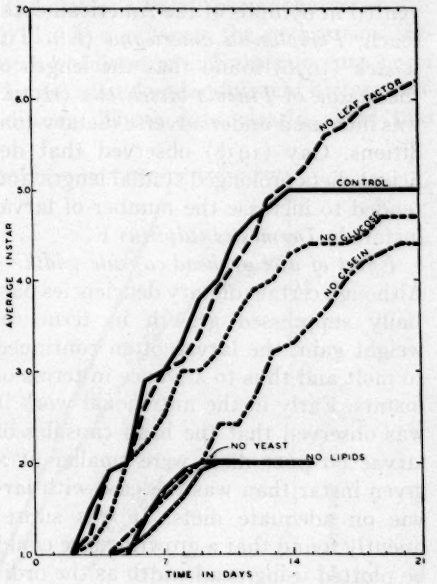


FIG. 1.—Effect of diet on instar progression of European corn borer larvae.

fifth instar, at which point molting did not stop, as in the case of the larvae receiving the basal diet, but continued at the previous rate. Mortality was high after the fifth stadium, and nearly all the insects were dead by the twenty-first day. No larvae reached maturity (pupation or diapause) on any of the deficient diets. On the basal diet nearly all had matured by the twenty-first day.

Many workers have observed that adverse nutritional or other culturing conditions tend to prolong the length of the larval life and to cause the appearance of supernumerary instars. However, the

author found few papers in which data on stadial duration were presented. Hodge (1933) found no significant effect of different food plants on the length of the various stadia in *Melanoplus differentialis* (Thos.). Gier (1947) noted that, under conditions of extreme dietary inadequacy, molting was delayed or prevented in nymphs of the American cockroach, *Periplaneta americana* (L.). Titschack (1926) found that the length of the stadia of *Tineola bisselliella* (Hum.) was increased under adverse dietary conditions. Gay (1938) observed that deficient diets prolonged stadial length and tended to increase the number of larval instars in *Dermestes vulpinus* F.

*Effect of diet on head-capsule width.*—Although certain dietary deficiencies partially suppressed growth in terms of weight gain, the larvae often continued to molt and thus to advance in terms of instars. Early in the nutritional work it was observed that the head capsules of larvae on poor diets were smaller at a given instar than was the case with larvae on adequate diets. It was subsequently found that a growth curve could be plotted using head width as the ordinate and instar number as the abscissa and that such growth curves reflected the same dietary differences as did curves involving weight and instar. Figure 2 shows such growth curves for the same cultures as those considered in Figure 1. It can be seen in Figure 2 that the larvae displaying the largest head width (left-hand ordinate) were also of the greatest weight (right-hand ordinate). The weights shown in the right-hand ordinate are those of the larvae at the beginning of the stadium, and no account could be made of the weight gained by fifth-instar larvae. This method of showing growth was disadvantageous to the control culture because the weight of larvae on the basal diet usually increased to nearly 100 mg. during the fifth stadium, whereas no such increase was observed on the other diets shown. It is of particular interest to note that the larvae on the casein-deficient diet reached the fifth stadium with a head-capsule width of less than the control third-instar larvae and with an average weight of only about 2 mg.

The relationship between head width and body weight was determined by weighing larvae within 24 hours after molting and at the same time measuring their head widths. By using various diets a large range of weights and widths was obtained. The results are presented in Figure 3. As shown in this figure, body weight plotted against head width on log-log graph paper yielded a straight line. Although not excessive, the deviation about the calculated regression line could probably have been reduced by making the measurements immediately following ecdysis rather than within 24 hours.

The correlation between head width and weight was constant, and data obtained from larvae on inadequate diets fell along the same regression line as did data from larvae on the basal diet. In short, it was found that the influence of diet on head width was the same as its influence on weight and that the two effects could not be separated. It follows from this relationship that the principal factor determining the width of the head capsule was the weight of the molting insect and not its instar number. This correlation implies simple allometric growth, the expression for which is $Y = bX^k$, or $\log Y = \log b + k \log X$. This growth equation has been applied to measurements of many animal forms, and its use is well reviewed by Huxley (1932) and by Reeve and Huxley (1945). It has been applied to problems in arthropod growth characteristics by several workers, and recently Newcombe (1949) and New-

combe *et al.* (1949) described simple allometric relationships between weight and linear dimensions in the blue crab, *Callinectes sapidus* Rathbun.

*Effect of diet on progression factors.*—Dyar (1890) postulated that the head-capsule width of a lepidopterous larva increases as a geometric progression from instar to instar and that the ratio of the head width of any given instar to the head width of the next instar is a constant (the progression factor), the value of which is a species characteristic. Progression factors are now usually expressed as the inverse of the original formulation by Dyar and are obtained by dividing the head width before molting into the head width after molting. Dyar suggested the application of this "Rule" as a means of detecting missing instars in life-history studies and also as a valid species characteristic.

Table 2 presents the progression factors obtained with European corn borer larvae reared under different dietary conditions. These data clearly show that

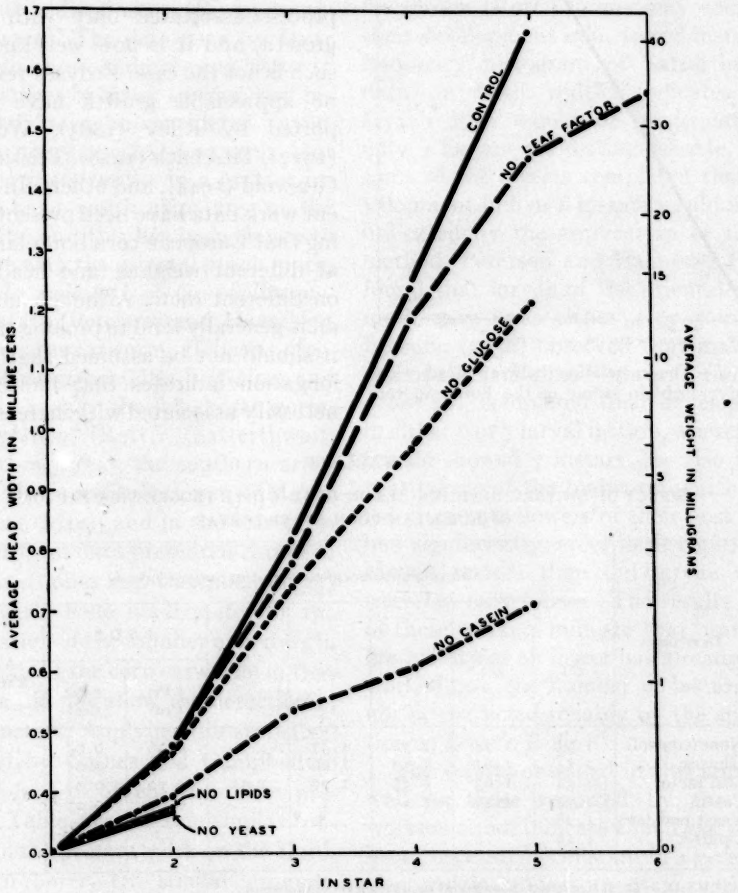


FIG. 2.—Growth of European corn borer larvae under different dietary conditions

the progression factors varied significantly from instar to instar, even on the basal diet, and in all cases declined in value from the second molt. It is also apparent from these data that dietary differences caused significant differences in the average (Dyar's) ratios.

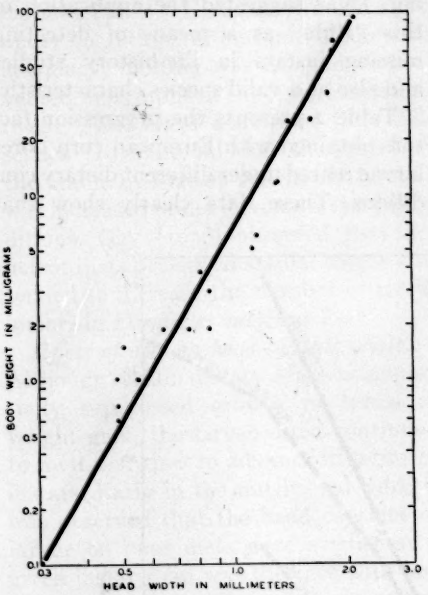


FIG. 3.—The correlation between head width and body weight in larvae of the European corn borer.

## DISCUSSION

The allometric relationship between body weight and head-capsule width does not in itself invalidate Dyar's Rule. Dyar's Rule would apply only if conditions causing poor growth prevented molting until sufficient growth had occurred to allow the postmolt head dimensions to show the increase "characteristic" of the species. In short, it would be necessary to assume that molting is a process associated only with absolute growth, and it is now well known that such is not the case. Ecdyses resulting in no appreciable growth have been reported by Riley (1883), Wodsedalek (1917), Titschack (1926), Teissier (1928), Griswold (1944), and others. In the present work data have been presented showing that European corn borer larvae molt at different weights (and head widths) on different diets. Although inadequate diets generally tend to prolong the stadia, it should not be assumed that this prolongation indicates that molting is by necessity associated with increase in size.

TABLE 2

EFFECT OF DIETARY DEFICIENCIES ON HEAD-WIDTH PROGRESSION FACTORS OF EUROPEAN CORN BORER LARVAE

| Deficiency | Head-width progression factor (instar/instar) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2/1 | 3/2 | 4/3 | 5/4 | 6/5 | L.S.D.* 1 Per Cent | L.S.D.* 5 Per Cent | Av. Ratio† |
| None (control) | 1.55 | 1.74 | 1.52 | 1.37 | ..... | 0.06 | 0.05 | 1.55 |
| Glucose | 1.55 | 1.63 | 1.30 | 1.26 | ..... | 0.09 | 0.05 | 1.44 |
| Leaf factor | 1.54 | 1.63 | 1.55 | 1.20 | 1.11 | 0.10 | 0.08 | 1.41 |
| Casein | 1.34 | 1.40 | 1.11 | ‡ | ..... | 0.07 | 0.05 | 1.28 |
| Yeast powder | 1.28 | ..... | ..... | ..... | ..... | ..... | ..... | ..... |
| Lipids | 1.25 | ..... | ..... | ..... | ..... | ..... | ..... | ..... |

* L.S.D. = Least significant difference at indicated level of probability.

† L.S.D. for average ratios: at 1 per cent, 0.04; at 5 per cent, 0.03.

‡ Too few specimens attained the fifth instar to be considered statistically.

A deficient diet may be expected to result in a greater or lesser slowing of many physiological processes as well as growth in size and weight.

In spite of this source of error, Dyar's Rule might be a useful tool under controlled environmental and nutritional culturing conditions except for the fact that in some species it has proved to be unreliable to determine the number of instars even under controlled conditions. The main objection has been that the supposedly constant factor is not actually constant but tends to decline in value in succeeding instars (Ripley, 1923; Calvert, 1929). The data used by Dyar (1890) also show a decreasing ratio in the later stages of many species, and his "constants" must be considered "averages." In many species, one molt (not always the first) results in a greater increase in head width than any of the other molts, and this has been shown to be the case with the oriental peach moth, *Laspeyresia molesta* ( = *Grapholita molesta* [Busck]) (Peterson and Haeussler, 1928); the corn earworm, *Heliothis obsoleta* ( = *H. armigera* [Hbn.]) (Gaines and Campbell, 1935); the black cutworm, *Agrotis ypsilon* (Rott.) (Satterthwait, 1933; Forbes, 1934); the southern army worm, *Prodenia eridania* (Cram.) (Mayer and Babers, 1944); and in the European corn borer from data presented earlier in this paper. Gaines and Campbell (1935) found Dyar's Rule inadequate for the determination of the number of instars in the life-cycle of the corn earworm, in that their data did not allow the detection of missing instars. Applying the statistical tests used by Gaines and Campbell to the data used to obtain the ratios presented in Table 2 resulted in similar conclusions in the present work on the European corn borer. On similar grounds, Mayer and Babers (1944) questioned its validity in the case of the southern army worm.

Griswold (1944) expressed doubt that Dyar's Rule was applicable to larvae of the clothes moth, *Tineola bisselliella* (Hum.) because of the variable number of instars (6–12) in the larval life of that insect. The results of Donohoe *et al.* (1949) in life-history studies of the raisin moth, *Ephestia figulilella* Greg., indicate from 4 to 8 instars and make it doubtful that Dyar's Rule would be of much value in determining the number of instars. Satterthwait (1933) observed that *Agrotis ypsilon* (Rott.) larvae may complete their development in 6, 7, or 8 instars. A frequency histogram of Satterthwait's data on head widths indicates that Dyar's Rule would be inadequate, as only 7 instars are distinguishable. That some of the insects completed their development in 6 or 8 instars would not be indicated by the application of such a method. Peterson and Haeussler (1928) found that larvae of the oriental peach moth may have either 4 or 5 instars. Lejeune (1950) observed that male larvae of the jack-pine budworm, *Choristoneura* sp., completed their development in either 6 or 7 larval instars, whereas the female showed 7 instars. He also found that larvae of the budworm which were fed staminate flowers of their host plant had significantly wider head capsules at certain instars than did larvae which were fed only foliage. The results of all of these workers indicate that, until the life-history of an insect has already been worked out, the number of instars cannot be predicted reliably by the application of Dyar's Rule.

The results obtained in this study, as well as those reported by the other workers cited, indicate that Dyar's ratio is not necessarily constant in a given species and is subject to great variation, owing to the dietary conditions under

which the insect is reared. It is apparent, therefore, that Dyar's Rule does not describe a fundamental characteristic of the growth of lepidopterous larvae.

## SUMMARY AND CONCLUSIONS

1. Dietary deficiencies may suppress or retard molting or may cause supernumerary molts in larvae of the European corn borer.

2. Diets causing suboptimal growth in terms of weight also cause suboptimal growth in terms of head-capsule width.

3. An allometric relationship is demonstrable between body weight and width of head capsule, independent of diet. The correlation between instar number and head-capsule width is subject to great variation, depending on nutritional conditions.

4. Head-width progression factors in the European corn borer are not constant from molt to molt but decline from the second molt and reflect differences caused by dietary deficiencies.

5. From the results obtained and from a consideration of literature on growth of lepidopterous larvae, it is concluded that Dyar's Rule has no fundamental basis.

## LITERATURE CITED

ABERCROMBIE, W. F. 1936. Studies on cell number and the progression factor in the growth of Japanese beetle larvae (*Popillia japonica* Newman). Jour. Morphol., **59**:91–112.

BECK, S. D.; LILLY, J. H.; and STAUFFER, J. F. 1949. Nutrition of the European corn borer, *Pyrausta nubilalis* (Hbn.). I. Development of a satisfactory purified diet for larval growth. Ann. Ent. Soc. Amer., **42**:483–96.

BECK, S. D., and STAUFFER, J. F. 1950. An aseptic method for rearing European corn borer larvae. Jour. Econ. Ent., **43**:4–6.

BODENHEIMER, F. S. 1933. The progression factor in insect growth. Quart. Rev. Biol., **8**:92–95.

BROOKS, W. V. 1886. Report on the stomatopoda collected by HMS Challenger during the years 1873–1876. Challenger Rept., **16**:Zoöl. Part 45.

CALVERT, P. P. 1929. Different rates of growth among animals with special reference to Odonata. Proc. Amer. Phil. Soc., **68**:227–74.

DONOHOE, H. C.; SIMMONS, P.; BARNES, D. F.; KALOOSTIAN, C. H.; FISHER, C. K.; and HEINRICH, C. 1949. Biology of the raisin moth. U.S.D.A. Tech. Bull. 994.

DYAR, H. G. 1890. The number of moults of lepidopterous larvae. Psyche, **5**:420–22.

FORBES, W. T. M. 1934. A note on Dyar's law. Bull. Brooklyn Ent. Soc., **29**:146–49.

GAINES, J. C., and CAMPBELL, F. L. 1935. Dyar's rule as related to the number of instars of the corn earworm, *Heliothis obsoleta* (Fab.), collected in the field. Ann. Ent. Soc. Amer., **28**:445–61.

GAY, F. J. 1938. A nutritional study of the larvae of *Dermestes vulpinus* F. Jour. Exper. Zoöl., **79**: 93–107.

GIER, H. T. 1947. Growth rate in the cockroach, *Periplaneta americana*. Ann. Ent. Soc. Amer., **40**:303–17.

GRISWOLD, G. H. 1944. Studies on the biology of the webbing clothes moth (*Tineola bisselliella* Hum.). Cornell Univ. Agr. Exper. Stat. Mem. 262.

HODGE, C. 1933. Growth and nutrition of *Melanoplus differentialis* Thomas (Orthoptera; Acrididae). I. Growth on a satisfactory diet and on diets of single food plants. Physiol. Zoöl., **6**:306–28.

HUXLEY, J. S. 1932. Problems of relative growth. New York: Dial Press.

LEJEUNE, R. R. 1950. The effect of jack-pine staminate flowers on the size of larvae of the jack-pine budworm, *Choristoneura* sp. Canad. Ent., **82**: 34–43.

LUDWIG, D. 1932. The effect of temperature on the growth curves of the Japanese beetle (*Popillia japonica*). Physiol. Zoöl., **5**:431–47.

———. 1934. The progression factor in the growth of the Japanese beetle (*Popillia japonica* Newman) (Coleop.; Scarabaeidae). Ent. News, **45**: 141–53.

MAYER, E. L., and Babers, F. H. 1944. Head capsule measurements of southern army worm larvae (*Prodenia eridania* [Cramer]). Ann. Ent. Soc. Amer., **37**:214–20.

NEWCOMBE, C. L. 1949. A method for studying growth in different groups of arthropods. Science, **109**:84–85.

NEWCOMBE, C. L.; CAMPBELL, F.; and ECKSTEINE, A. M. 1949. A study of the form and growth of the blue crab, *Callinectes sapidus* Rathbun. Growth, **13**:71–96.

PETERSON, A., and HAEUSSLER, G. J. 1928. Some observations on the number of larval instars of the oriental peach moth. *Laspeyresia molesta* Busck. Jour. Econ. Ent., **21**:843–52.

PRZIBRAM, H., and MEGUSAR, P. 1912. Wachstumsmessungen an *Sphodromantis bioculata* Burm. I. Lange und Masse. Arch. f. Entw'mech., **34**:680–741.

REEVE, E. C. R., and HUXLEY, J. S. 1945. Some

problems in the study of allometric growth. In: Essays on growth and form presented to D'Arcy Wentworth Thompson. Edited by W. E. Le Gros Clark and P. B. Medawar, pp. 121–56. Oxford: Oxford University Press.

RILEY, C. V. 1883. Number of moults and length of larval life as influenced by food. Amer. Nat., 17:547–48.

RIPLEY, L. B. 1923. The external morphology and postembryology of noctuid larvae. Illinois Biol. Mono., 8:1–102.

SATTERTHWAIT, A. F. 1933. Larval instars and feeding of the black cutworm, *Agrotis ypsilon* Rott. Jour. Agr. Res., 46:517–30.

TEISSIER, G. 1928. Sur l'indépendance relative de la croissance et des mues chez les insectes. Compt. rend. Soc. biol., 98:903–5.

TITSCHACK, E. 1926. Untersuchungen über das Wachstum, den Nahrungsverbrauch, und die Eierzeugung. II. *Tineola bisselliella* Hum. Gleichzeitig ein Beitrag zur Klarung der Insecthautung. Zeitschr. f. wissensch. Zoöl., 128:569–69.

TRAGER, W. 1935. The relation of cell size to growth in insect larvae. Jour. Exper. Zoöl., 71:489–508.

WODSEDALEK, J. E. 1917. Five years of starvation of larvae. Science, 46:366.

# SOCIAL DOMINANCE AND RECEPTIVITY IN THE DOMESTIC FOWL[1]

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IN RECENT years there has been much interest in the psychological aspects of reproductive behavior in birds, especially with regard to display behavior (Armstrong, 1947). One phase of these studies has been concerned with the relation of dominance status in the group or between the sexes to mating behavior.

Among chickens the most dominant of the cocks tends to be most successful in mating (Guhl, Collias, and Allee, 1945) and to sire the most chicks (Guhl and Warren, 1946). In one flock the lowest-ranking male was completely inhibited from mating, even in the absence of his superiors, although he mated readily with strange hens. A similar influence of conditioned behavior associated with dominance relationships was found in hens treated with an androgen. These hens showed a marked increase in aggressiveness when placed with a small flock of strange hens but resumed the behavior characteristic of their subordinate rank upon return to their home pen (Guhl, Collias, and Allee, 1945). In tests made with hormonally treated capons it was concluded that passive dominance of males over females facilitated mating (Guhl, 1949).

Murchison (1935) tested cockerels and pullets in a discrimination cage and concluded "that male discriminations for pairs of males are away from dominance, and that female discriminations for pairs of males are in the direction of dominance." A similar discrimination test, using adult birds, failed to substantiate this conclusion (Guhl, 1942). Interest in this aspect of behavior was stimulated by the assumption that if dominant hens mated more readily with dominant males, there might be a progressive increase in aggressiveness in successive generations.

Schjelderup-Ebbe (1935) noted that high rank among females interfered with mating. More recently, in several small flocks under extended observation it was found that there was a consistent tendency toward a negative correlation be-

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tween the frequency at which hens were mated and their rank in the social order (Guhl, Collias, and Allee, 1945).

In an attempt to determine experimentally the influence of high social rank upon the receptivity of hens, the following procedure was used.

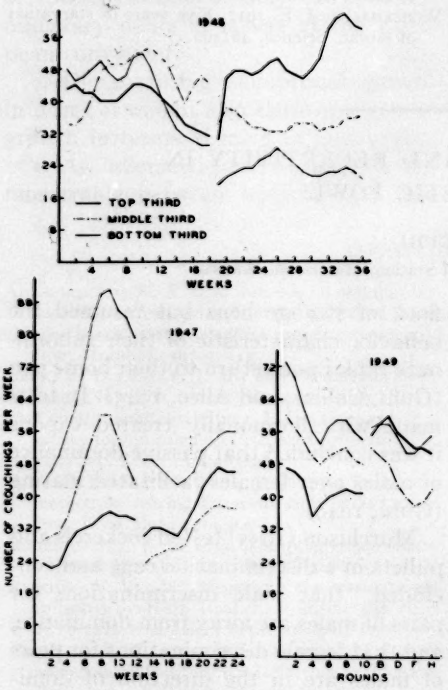


FIG. 1.—The rates at which hens, as groups, in each of three levels of the dominance orders, crouched receptively for males, before and after subflocking to alter the degree of domination and submission.

## PROCEDURE

Observations were made on three flocks of White Leghorns. The 1946 flock was composed of birds from the Kansas State College flock; the 1947 flock contained birds originating from five different sources; and the 1949 birds were from the second filial generation of the 1947 flock, following selective breeding for high and low aggressiveness. The peck-order in each flock was determined, and dominance relations were recorded throughout the experiment.

Three cocks were used in 1946 and 1947 and five in 1949. These males were caged, as a group, in such a manner that the females and the males were visible to one another. Each day the males were placed singly and successively in the pen of the hens for 21 minutes each in 1946 and 1947, and for 18 minutes each in 1949. The order in which the cocks were used followed a system of rotation. The observations were made for 18 weeks and 12 weeks and for 10 rounds (of 5 days each) for the respective years.

At the approximate peak of the laying cycle, each flock was subdivided into three levels in the dominance order. The males were rotated among these three pens. Only one male was used with each of the subflocks on a given day. At the close of each daily observation the males were shifted to a cage which placed them in view of the hens with which they would be released during the next day. The length of the observation periods and the number of weeks during which this procedure was followed were essentially the same as those prior to subflocking. For 1949 the data were summarized by rounds and not by weeks. After subflocking, three of the five males were used, and the data are given for double rounds (6 days) and indicated by letters in Figure 1.

Although records were made of courting by the males, avoidance of the male by the females, the crouching of females in response to courting, and mating, this report is concerned primarily with the data on crouching. The frequency of crouching is used here as a measure of receptivity. A grand total of 10,587 crouches was used in the following analysis.

## RESULTS

The variability in the frequency of crouching among the hens, as individuals, in each of the three levels of the peck-orders is given in Table 1. The figures are given separately for this behavior in the large flocks and in subflocks. The figures given under "Range" are totals for the sets of weeks (or rounds for 1949) during which observations were made. Hens displaying relatively low or high receptivity were found in all levels of the dominance orders. Standard deviations are approximately 50 per cent of the means in 12 of the 18 levels given. In all three large flocks the mean rates of crouching by the top-level hens were the lowest and those of the bottom levels were the highest. Subsequent to subflocking, the high-ranking hens crouched more frequently than, or approximately as often as, those hens composing the lowest level. Similar changes in receptivity are also evident in data on the range of frequencies.

TABLE 1

VARIABILITY IN TOTAL NUMBER OF CROUCHES BY INDIVIDUAL HENS IN EACH OF THREE LEVELS OF DOMINANCE ORDERS, FOR TOTAL OBSERVATIONS DURING EACH SET OF WEEKS (ROUNDS FOR 1949)

| Level | n | Range | Mean | Standard Deviation |
|---|---|---|---|---|
| | | 1946 | | |
| *Large flock:* | | | | |
| Top | 11 | 21–110 | 65 | 29.6 |
| Middle | 11 | 15–134 | 74 | 36.7 |
| Bottom | 10 | 10–125 | 76 | 38.8 |
| *Subflocks:* | | | | |
| Top | 11 | 33–239 | 81 | 36.2 |
| Middle | 11 | 24–126 | 56 | 28.6 |
| Bottom | 10 | 11– 73 | 41 | 19.8 |
| | | 1947 | | |
| *Large flock:* | | | | |
| Top | 10 | 10– 77 | 37 | 23.3 |
| Middle | 9 | 20–161 | 60 | 39.4 |
| Bottom | 13 | 0–120 | 65 | 23.9 |
| *Subflocks:* | | | | |
| Top | 10 | 20– 94 | 45 | 20.9 |
| Middle | 9 | 24–117 | 41 | 31.5 |
| Bottom | 13 | 5– 98 | 46 | 32.9 |
| | | 1949 | | |
| *Large flock:* | | | | |
| Top | 9 | 28– 84 | 42 | 16.5 |
| Middle | 9 | 27–123 | 52 | 31.7 |
| Bottom | 9 | 42–112 | 60 | 22.7 |
| *Subflocks:* | | | | |
| Top | 9 | 20– 94 | 54 | 22.7 |
| Middle | 9 | 11– 89 | 44 | 27.0 |
| Bottom | 9 | 18– 82 | 55 | 19.5 |

Of 118 hens used in the three flocks, 27 died or had to be removed. Sudden changes in temperature to extremes over 100° F. and the extrusion of the oviduct (prolapse) caused the death rate to be high in the 1946 and 1949 flocks. As individuals which were relatively high or low in receptivity were found in all levels of the social order, the analytical comparisons will be based on the data of those birds which survived the full experiment each year.

The data on crouching for groups of hens are presented as graphs (Fig. 1), in which the lines have been smoothed with a five-point moving average. Two different analyses were made. One was vertical, or between each pair of the three levels before and after subflocking (Table 2). The other, or horizontal analysis, was made between a given social level as part of a large flock and the same individuals as a subflock (Table 3). In each comparison the significance of the difference between the means of weekly rates of crouching was determined.

In the 1946 flock there were no statistically significant differences in crouching among the three social levels in the large flock (Table 2; 1–18 weeks in Fig.

## TABLE 2

### INTERLEVEL COMPARISONS OF WEEKLY RATES AT WHICH HENS, AS GROUPS, IN EACH SOCIAL LEVEL CROUCHED FOR MALES

| Social Levels Compared (n) | Large Flock | | Subflock | |
|---|---|---|---|---|
| | Means | Difference | Means | Difference* |
| | 1946 | | | |
| Top (11):middle (11) | 40:45 | − 5 | 49:34 | +15† |
| Top (11):bottom (10) | 40:42 | − 2 | 49:22 | +17† |
| Middle (11):bottom (10) | 45:42 | + 3 | 34:22 | +12† |
| | 1947 | | | |
| Top (10):middle (9) | 31:45 | −14† | 38:31 | + 7‡ |
| Top (10):bottom (13) | 31:70 | −39† | 38:50 | −12† |
| Middle (9):bottom (13) | 45:70 | −25† | 31:50 | −19† |
| | 1949 | | | |
| Top (9):middle (9) | 42:52 | −10‡ | 54:44 | +10‡ |
| Top (9):bottom (9) | 42:60 | −18† | 54:55 | − 1 |
| Middle (9):bottom (9) | 52:60 | − 8 | 44:55 | −11† |

* No dagger indicates no statistical significance.
† Shows significance beyond the 1 per cent level.
‡ Shows significance beyond the 5 per cent level.

## TABLE 3

### COMPARISON OF WEEKLY RATES AT WHICH HENS, AS A GROUP, IN A GIVEN SOCIAL LEVEL CROUCHED AS MEMBERS OF LARGE FLOCK AND AS SUBFLOCK

| Flocks | Top Level | | | Middle Level | | | Bottom Level | | |
|---|---|---|---|---|---|---|---|---|---|
| | n | Means | Difference* | n | Means | Difference* | n | Means | Difference* |
| | 1946 | | | | | | | | |
| Large | 11 | 40 | − 9† | 11 | 45 | +11† | 10 | 42 | +20† |
| Subflocks | 11 | 49 | ..... | 11 | 34 | ..... | 10 | 22 | ..... |
| | 1947 | | | | | | | | |
| Large | 10 | 31 | − 7 | 9 | 45 | +14† | 13 | 70 | +20† |
| Subflocks | 10 | 38 | ..... | 9 | 31 | ..... | 13 | 50 | ..... |
| | 1940 | | | | | | | | |
| Large | 9 | 42 | −12† | 9 | 52 | + 8 | 9 | 60 | + 5 |
| Subflocks | 9 | 54 | ..... | 9 | 44 | ..... | 9 | 55 | ..... |

* No dagger indicates no statistical significance.
† Shows significance beyond the 1 per cent level.

1), but the differences were highly significant after subflocking (Table 2; 19–36 weeks in Fig. 1). In the 1947 flock the differences were highly significant for both sets of weeks (1–12 and 13–24 weeks). In 1949 there was no significant difference between the middle and bottom levels in the large flock or between the top and bottom subflocks. The remaining comparisons showed high significance.

As the curves in Figure 1 show, the hens in the top third of each flock crouched more often as subflocks than they did as habitually dominating members of the upper level in the large flocks. These differences were statistically significant for the 1946 and 1949 flocks, but not for 1947 (Table 3). Contrariwise, the other two levels in each flock crouched less frequently as subflocks, and these differences were statistically significant in the 1946 and 1947 flocks, but not in 1949.

## DISCUSSION

The experimental animals consisted of three flocks of White Leghorns, with, presumably, different genetic backgrounds, which showed considerable individual variation in receptivity. By means of subflocking, the degree of domination and/or subordination was altered. The hens composing the middle and bottom thirds of the dominance order crouched less often as members of subflocks than as member of a large flock. This result was expected, as the total time each female spent in the presence of a male was less in subflocks than in the original flock. However, the hens composing the top social level crouched more often when they had fewer hens to dominate. This difference between the top social level and the other two levels may be attributed to a decrease in the degree of domination subsequent to subflocking.

The results substantiate, by experimental methods, the observation made by Schjelderup-Ebbe (1935) that female despotism hinders pairing. The reduction in receptivity also explains, in part, the consistent negative (although not always statistically significant) correlation between social status and the frequency of giving the sexual crouch as found by Guhl, Collias, and Allee (1945).

In a review of bird behavior by Armstrong (1947), evidence is given of a general agreement that a number of psychological factors serve to synchronize the sexes. Although some of these are also applicable to chickens, this report shows that the habit of dominating exerts a suppressive influence on the mating behavior of hens. This observation would be of greater interest if it were also demonstrated in a free-ranging flock of polygamous birds in which the females possess a dominance order.

The writer considered the possibility that the habit of submission among low-ranking hens might augment their rate of crouching. However, some of these hens were harassed and spent much time in neutral areas or showed signs of inanition. These conditions are associated with sustained avoidance and reduce the chances of these hens being in the proximity of the male; nevertheless, the data (Fig. 1) do suggest such a possibility.

No attempt was made to determine the fertility or hatchability of the eggs obtained. It does not necessarily follow, however, that high-ranking hens would have fewer chicks as a consequence of an obstruction to mating by their habit of dominating. There is the suggestion that very aggressive hens might be mated more frequently in relatively small flocks than in large flocks.

## SUMMARY

The influence of the habit of dominating associated with high rank in the social

hierarchy upon receptivity in hens was determined experimentally. Relative dominance was altered by subdividing each of three flocks into three levels of the dominance orders. The rates at which hens in each level, as groups, crouched submissively for cocks were used as measures of receptivity. Hens composing the top level crouched less often than those of the middle or bottom levels, and those of the bottom level crouched at the highest rates. Subsequent to subflocking, the high-ranking hens crouched more frequently or approximately as often as those composing the lowest level.

## LITERATURE CITED


ARMSTRONG, E. A. 1947. Bird display and behaviour. London: Lindsay Drummond.

GUHL, A. M. 1942. Social discrimination in small flocks of the common domestic fowl. Jour. Comp. Psychol., 34:127–48.

———. 1949. Heterosexual dominance and mating behavior in chickens. Behaviour, 2:106–20.

GUHL, A. M.; COLLIAS, N. E.; and ALLEE, W. C. 1945. Mating behavior and the social hierarchy in small flocks of White Leghorns. Physiol. Zoöl., 17:365–90.

GUHL, A. M., and WARREN, D. C. 1946. Number of offspring sired by cockerels related to social dominance in chickens. Poultry Sci., 25:460–72.

MURCHISON, C. 1935. The experimental measurement of a social hierarchy in *Gallus domesticus*. II. The identification and inferential measurement of social reflex No. 1 and social reflex No. 2 by means of social discrimination. Jour. Sociol. Psychol., 6:3–30.

SCHJELDERUP- EBBE, T. 1935. Social behavior in birds. In: MURCHISON, C., Handbook of social psychology, chap. xx, pp. 947–72. Worcester, Mass.: Clark University Press.

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